TRAIL-induced kinases activation and apoptosis: towards improved death receptor targeted therapy for lung cancer

Kaamar Azijli

The research in this thesis was performed within the framework of Dutch Top Institute Pharma, project T3-112: "TNF-ligands in cancer".

The experiments were performed at the Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands.

Publication of this was financially supported by:
Amgen, Roche Pharmaceuticals B.V., Bio-Greiner B.V., Tebu-Bio, Universitair Medisch Centrum Groningen, Rijksuniversiteit Groningen.

Printed by: Ipskamp Drukkers

ISBN: 978-90-367-6110-9

rijksuniversiteit groningen

TRAIL-induced kinases activation and apoptosis: towards improved death receptor targeted therapy for lung cancer

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 17 april 2013
om 14:30 uur

door

Kaamar Azijli

geboren op 14 september 1984
te Amsterdam

Promotores	:	Prof. dr. F.A.E. Kruyt Prof. dr. G.J. Peters Prof. dr. S. de Jong
Beoordelingscommissie	:	Prof. dr. E.G.E. de Vries Prof. dr. W. Quax Prof. dr. H. Groen

“In order to be irreplaceable one must always be different”.

- Coco Chanel -

For my Loved Ones

Contents

Chapter 1

General introduction and Outline of the thesis

GENERAL INTRODUCTION

Cancer is one of the leading causes of death across the world. Recently, it has overtaken heart disease from its number one position as the world's most deadly disease. Among the different cancer types, lung cancer is one of the most prevalent ones, with each year approximately 1.1 million patients succumbing to this devastating disease [1]. The main cause of lung cancer is exposure to tobacco smoke, which accounts for 85% of all lung cancer cases. Radon gas, asbestos and genetic factors are also causes of lung cancer development. Lung cancer can be divided into two histological subtypes, namely small cell lung cancer (SCLC), which represents 15-20% of all lung carcinomas and non-small cell lung cancer (NSCLC), which accounts for 80-85% of all lung cancer cases. NSCLC can be divided into three major types, i.e. squamous cell carcinoma, adenocarcinoma and large-cell carcinoma [2].

NSCLC is often diagnosed at an advanced stage of disease contributing to a poor prognosis. At early stages of the disease, i.e. in the absence of metastases, removal of the primary tumor by surgery gives rise to relatively good prognosis. The median 5-year survival rate is 50 to 60% for local stage 1A and 40% for stage 1B. At later stages with local or distant metastases cure rates drop drastically. The five-year overall survival of stage IIIB NSCLC is 5-8% and for patients with stage IV it is only 1% [3]. The first-line treatment of advanced NSCLC is platinum-based chemotherapy, such as cisplatin or carboplatin in combination with a third generation cytotoxic drug, gemcitabine, pemetrexed, paclitaxel, docetaxel, etoposide or vinorelbine. However, only partial responses can be achieved in just 30-40% of the cases and severe side effects are often observed [4]. In recent years a lot of progress has been made with the introduction of targeted therapy for NSCLC patients. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib (Iressa) and erlotinib (Tarceva) proved to be quite effective, but only in a small group of patients; Japanese women, non-smokers and patients with adenocarcinoma. These patients carry specific activating mutations in the tyrosine kinase domain of the EGFR leading to constitutive activation of this pathway, and which correlated with clinical response to EGFR inhibitors [5;6]. Recently, the anaplastic lymphoma kinase (ALK) tyrosine kinase (TK) receptor has emerged as a relevant biomarker and therapeutic target in NSCLC. ALK inhibitors, such as crizotinib, will probably benefit only a subgroup of NSCLC patients with an EML4-ALK fusion gene [7;8]. Despite these promising advancements in the treatment of groups of NSCLC patients, for many NSCLC patients new treatment approaches need to be discovered.

Apoptosis-targeted therapy provides an interesting strategy for the treatment of various cancers, including lung cancer. In particular, TNF-related apoptosis-inducing ligand (TRAIL), which belongs to the Tumor Necrosis Factor (TNF) superfamily, is a promising cancer therapeutic as it targets tumor cells in a selective way through specific TRAIL receptors. Currently, different TRAIL receptor targeting agents are being evaluated in

clinical Phase I/II studies in different tumor types. Phase I studies have shown that patients tolerate recombinant TRAIL (Apo2L/dulanerim) as well as agonistic antibodies quite well [9].

TRAIL can bind to five different receptors, the death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), and the soluble receptor osteoprotegerin (OPG) [10]. TRAIL activates the extrinsic pathway by binding to TRAIL-R1 and TRAIL-R2. The death inducing signaling complex (DISC) consisting of FADD and caspase-8 is than formed, leading to caspase-8 and caspase-3 activation followed by apoptosis. Caspase-8 can also cleave the pro-apoptotic Bcl-2 family member Bid into tBid, which in turn results in activation of other pro-apoptotic family members, such as BAX or BAK. This leads to the induction of mitochondrial activator outer membrane permeabilization (MOMP) and release of cytochrome c and Smac/Diablo in the cytosol, resulting in the activation of apoptosis through the intrinsic pathway that involves caspases-9 and -3 [11;12].

However, in addition to apoptosis induction that can be regarded as the canonical route of TRAIL signaling an increasing number of studies have shown that TRAIL can also activate diverse other intracellular non-canonical signaling pathways in tumor cells. These include mitogen activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K) and Akt and nuclear factor κB (NF-κB) that can enhance cell survival, cell proliferation and even migration/ invasion of tumor cells, thus possibly stimulating tumorigenesis [13;14]. Moreover, a large number of cancer cells, especially the highly malignant ones, are resistant to TRAIL-induced apoptosis [15]. Some cancer cells that were initially sensitive to TRAIL can also develop resistance. TRAIL resistance is related to alterations at different points in the signaling pathway, mutations in the death receptors, inhibition of active DISC formation by cFLIP or more downstream by overexpression of anti-apoptotic proteins amongst others XIAP and Bcl-2 family members. The activation of the survival pathways aforementioned can also counteract the apoptotic effect of TRAIL. To circumvent TRAIL resistance and to increase the efficacy of this molecule, TRAIL can be combined with other anti-cancer agents, such as kinase inhibitors, radiation and chemotherapy (see for extensive overview of TRAIL sensitizing strategies in lung cancer [16]).

The aim of the research described in this thesis is to obtain more knowledge of the molecular mechanisms governing TRAIL resistance in NSCLC, in particular the role of kinases herein. In addition, we have explored several combination treatments for enhancing TRAIL efficacy. Ultimately, the work may lead to the identification of novel predictive markers and therapeutic approaches for optimizing TRAIL-based therapies.

OUTLINE OF THE THESIS

As TRAIL-induced apoptosis is frequently blocked in tumor cells and even TRAIL-dependent tumor promoting events may occur, it is important to obtain more knowledge of the underlying mechanisms causing these unwanted phenomena. In this thesis the focus is on examining whether kinases and which type of kinases are involved in TRAIL resistance. In **Chapter 2** an overview is given of the different non-canonical signaling cascades that have been found to be triggered by TRAIL in cancer cell models representing various tumor types. Some kinases have been identified that enhance TRAIL-induced apoptosis in sensitive tumor cells such as Mitogen Activated Protein (MAP) kinases p38 and JNK. However, multiple other kinases have been found to contribute to non-apoptotic signaling in TRAIL resistant tumor cells, including IκB and PI3K/Akt. Yet other kinases such as the ROCK/ LIM kinase have been found to stimulate invasion. These different TRAIL inducible kinases are reviewed in detail in this chapter. In **Chapter 3** we have studied the activation of p38 and JNK by TRAIL in sensitive and resistant NSCLC cells and the mechanism and consequences of activation have been evaluated. Previously, it has been shown that TRAIL activates these two kinases through the formation of the secondary complex, which consists among others of FADD, TRADD, Caspase-8, FADD, TRAF2, and RIP1 [17]. In NSCLC cells the pro-apoptotic or anti-apoptotic effects of p38 and JNK activation by TRAIL were studied using selective chemical kinase inhibitors. The molecular mechanisms have been examined using siRNA-dependent knockdown and ectopic overexpression strategies. In particular, the involvement of RIP1 and caspase-8 in the activation of these pathways was investigated by silencing RIP1 expression with short hairpin (sh)RNA and using NSCLC H460 cells stably overexpressing the caspase-8 inhibitor CrmA, respectively. In **Chapter 4** we employed peptide arrays containing 1,024 different kinase pseudosubstrates as a kinomic approach to contrast kinase activation patterns in TRAIL apoptosis sensitive and resistant NSCLC cells. In this way, we also attempted to identify the kinases responsible for TRAIL-induced migration and invasion that we observed in TRAIL resistant NSCLC cells. A novel non-canonical TRAIL signaling route was revealed involving RIP1-Src-STAT3 signaling, which is preferentially activated by TRAIL-R2. We propose that combined use of TRAIL with selective kinase inhibitors will either overcome TRAIL resistance and/ or prevent TRAIL-induced cell migration.

In addition to examining TRAIL-induced protein kinase signaling and the application of protein kinase targeted inhibitors for enhancing TRAIL-dependent apoptosis we also explored other combination strategies that indirectly affect kinases. An agent that targets heat shock proteins and a novel chemotherapeutic targeting thymidylate synthase (TS) that causes DNA damage through its incorporation into DNA were examined for potentiating the apoptosis-inducing capacity of TRAIL. In **Chapter 5** we examined the combined anti-tumor effects of TRAIL together with a the novel anti-cancer agent, 17-

AAG, that targets Hsp90. Hsp90 is a potential target that plays an important role in the tumorigenesis of NSCLC [18]. Of note, it interacts and stabilizes several key signaling proteins/ kinases, like Akt, ErbB2, c-Met, and Raf-1. **Chapter 6** describes the synergistic effect of TRAIL with the novel chemotherapeutic TFT, a thymidylate synthase (TS) inhibitor that interferes with thymidylate production and in its triphosphate form can be incorporated into the DNA causing DNA damage. Underlying mechanisms of synergy were examined by studying the cell-cycle kinases, the intrinsic and extrinsic apoptotic pathways and TRAIL-R1 and TRAIL-R2 expression levels after TFT exposure. TFT is degraded by thymidine phosphorylase (TP), leading to strong decreases in the bioavailability of TFT. TP is often overexpressed in human cancers and was also found to play a role in the stimulation of angiogenesis, resulting in blood vessel formation sustaining tumor progression. The exact molecular mechanism of how TP stimulates angiogenesis is not fully clear. It is hypothesized that thymidine-derived sugars might play a role in this process. TP catalyzes the conversion of thymidine (TdR to thymine and deoxyribose-1-phosphate (dR-1-P), which can be converted to dR-5-P, glyceraldehydes-3-phosphate (G3P) or deoxyribose (dR). We characterized the thymidine-derived sugars that are related with the angiogenesis induction by TP, which is described in **Chapter 7**. Furthermore, subcellular localization of thymidine-derived sugars and whether they are secreted by the cancer cells were determined. Finally, a summary of the work presented in this thesis is provided and discussed in **Chapter 8**, including a view on the future perspectives of TRAIL therapy. A summary in Dutch concludes this thesis and can be found in **Chapter 9.**

Reference List

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60(5):277-300.
2. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359(13):1367-1380.
3. Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2007; 2(8):706-714.
4. Blackstock AW, Govindan R. Definitive chemoradiation for the treatment of locally advanced non small-cell lung cancer. J Clin Oncol 2007; 25(26):4146-4152.
5. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350(21):2129-2139.
6. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304(5676):1497-1500.
7. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008; 14(13):4275-4283.
8. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448(7153):561-566.
9. Wu GS. TRAIL as a target in anti-cancer therapy. Cancer Lett 2009; 285(1):1-5.
10. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev 2003; 14(3-4):337-348.
11. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003; 10(1):66-75.
12. Kruyt FA. TRAIL and cancer therapy. Cancer Lett 2008; 263(1):14-25.
13. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39(7-8):1462-1475.
14. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
15. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005; 12(3):228-237.
16. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
17. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
18. Gallegos Ruiz MI, Floor K, Roepman P, Rodriguez JA, Meijer GA, Mooi WJ et al. Integration of gene dosage and gene expression in non-small cell lung cancer, identification of HSP90 as potential target. PLoS One 2008; 3(3):e0001722.

Chapter 2

Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family

Kaamar Azijli, Birgit Weyhenmeyer, Godefridus J. Peters, Steven de Jong, Frank A.E. Kruyt

Cell Death & Differentiation (Accepted, pending revisions)

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-based therapy is currently evaluated in clinical studies as a tumor cell selective pro-apoptotic approach. However, besides activating canonical caspase-dependent apoptosis by binding to TRAIL-specific death receptors, the TRAIL ligand can activate non-canonical cell survival or proliferation pathways in resistant tumor cells through the same death receptors, which is counterproductive for therapy. Even more, recent studies indicate metastases promoting activity of TRAIL. In this review, the remarkable dichotomy in TRAIL signaling is highlighted. An overview of the currently known mechanisms involved in non-canonical TRAIL signaling and the subsequent activation of various kinases is provided. These kinases include RIP1, IκB/ NF-κB, MAPK p38, JNK, ERK1/2, MAP3K TAK1, PKC, PI3K/Akt and Src. The functional consequences of their activation, often being stimulation of tumor cell survival and in some cases enhancement of their invasive behavior are discussed. Interestingly, the non-canonical responses triggered by TRAIL in resistant tumor cells resemble that of TRAIL-induced signals in non-transformed cells. Better knowledge of the mechanism underlying the dichotomy in TRAIL receptor signaling may provide markers for selecting patients that will likely benefit from TRAIL-based therapy and could provide a rationalized basis for combination therapies with TRAIL death receptor targeting drugs.

Key Words: TRAIL, non-apoptotic, kinases, metastasis, RIP1

Abbreviations:

ADAM, a disintegrin and metalloproteinase; c-Cbl, Casitas B-lineage lymphoma; cFLIP, cellular flice-like inhibitory protein; DISC, death-inducing signalling complex; ECs, endothelial cells; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular regulated kinases; FADD, Fas-associated protein with death domain; HER, human epidermal receptor; IGF1R, insulin-like growth factor type 1 receptor; JNK, Jun NH2 terminal kinases; MAPK, Mitogen-activated protein kinases; Mst1, mammalian sterile 20-like kinase 1; mTOR, mammalian target of rapamycin; NEMO, NF-κB essential modulator; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; OPG, osteoprotegerin; PI3K, Phosphatidylinositide 3-kinases; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; RIP1, receptor interacting protein kinase 1; RIP3, receptor interacting protein kinase 3; ROCK, Rho kinase; ROS, reactive oxygen species; SEK1, stress-activated protein/ERK kinase 1; SFK, Src family kinases; SMAC, second mitochondria-derived activator of caspase; Src, Rous sarcoma oncogene cellular homolog; TAK1, transforming growth factor-β (TGF-β)-activated kinase 1; TGF-β, transforming growth factor-β; TRADD, TNF-receptor-associated death domain protein; TRAF2, TNF receptor associated factor 2; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; uPA, urokinase-type plasminogen activator; VSMCs, vascular smooth muscle cells; XIAP, X-linked inhibitor of apoptosis protein.

FACTS

- Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors are attractive targets for anticancer therapy due to their selective ability to mediate caspase-dependent apoptosis in tumor cells upon ligand binding without harming healthy tissues.
- Different TRAIL receptor-targeted agents have been developed showing promising antitumor activity in preclinical models, and several receptor agonists are being evaluated for activity in clinical studies.
- Resistance to the apoptosis-inducing effect of TRAIL receptor agonists is frequently encountered in tumor cells and can often be bypassed by combined treatments with radiotherapy and/ or chemotherapy.
- Contrasting its apoptotic activity, in TRAIL resistant tumor cells as well as in normal non-transformed tissue cells, TRAIL can activate non-apoptotic (non-canonical) signals resulting in the activation of various kinases that can enhance the proliferation, survival, migration/ invasion and angiogenic properties in a cell type-dependent manner.

OPEN QUESTIONS

- What is/ are the molecular mechanism(s) underlying the dichotomy in TRAIL signaling in sensitive versus resistant tumor cells?
- What is the molecular basis of the apparent differences between TRAIL-R1- and TRAIL-R2-dependent signaling?
- Which factors produced by the tumor microenvironment determine the outcome of TRAIL signaling and through what mechanism(s)?
- Can possible identified mechanism(s)/ proteins that function as an apoptotic switch in the TRAIL pathway be used as a target for developing therapeutic strategies for sensitizing tumor cells?
- Can TRAIL apoptosis sensitivity in tumor cells be predicted by not yet identified biomarkers, allowing the preselection of patients eligible for TRAIL receptor agonistic therapy?

INTRODUCTION

The death ligand TRAIL induces apoptosis in a wide variety of tumors without harming normal cells [1-3]. Moreover, its killing effect is irrespective of the proliferation status or tumor suppressor p53 status of cancer cells. These properties make TRAIL receptor-targeted therapy a very attractive anticancer approach.

The mechanisms by which TRAIL induces programmed cell death or apoptosis in cancer have been intensively investigated. TRAIL activates apoptosis via two membrane receptors, designated TRAIL-R1 (DR4) and TRAIL-R2 (DR5), whereas TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and circulating osteoprotegerin (OPG) probably function as competitive inhibitors in the regulation of TRAIL-induced apoptosis, because their lack of apoptosis-inducing capacity [4;5]. However, there is no clear correlation between DcR expression and TRAIL resistance, but almost all these studies relied on mRNA expression [6-8]. How widespread surface expression of the TRAIL receptors is in tumor cells and normal cells and how these receptors modulate TRAIL signaling is therefore not fully understood.

A number of TRAIL receptor targeting agents have been developed, including preparations of recombinant human soluble TRAIL (rhTRAIL) and derived variants, and agonistic monoclonal antibodies selective for either TRAIL-R1 or TRAIL-R2 [9-11]. High affinity and selective binding of either TRAIL-R1 or TRAIL-R2 by these receptor-selective agents together with reduced binding to decoy receptors is expected to enhance antitumor activity. However, currently it is unclear whether it will be more beneficial to target either TRAIL-R1 or TRAIL-R2 for optimal treatment, which may also vary in a tumor cell specific way. For example, TRAIL signals its cell death function through TRAIL-R1 in pancreatic cancer cells [12], and chronic lymhocytic leukemia [13], while in glioblastoma [14], colon and breast cancer cell lines [15] apoptosis induced by TRAIL goes via TRAIL-R2.

TRAIL apoptotic signaling is initiated following ligand binding to TRAIL receptors and subsequent recruitment of the adapter protein Fas-associated protein with death domain (FADD) and the cystein-aspartic protease procaspase-8, leading to the formation of a complex named the death-inducing signaling complex (DISC), which promotes caspase-8 activation and further downstream caspase-3 activity, ultimately leading to cell death [1;4;5;16] (see Figure 1). Cellular flice-like inhibitory protein (cFLIP), a non-functional procaspase-8 homologue, can compete with procaspase-8 for FADD binding leading to apoptosis suppression. Full activation of this so called extrinsic apoptosis pathway often requires the cross-activation of intrinsic or mitochondrial apoptosis that is mediated by caspase-8-dependent cleavage of pro-apoptotic Bcl-2 family member Bid and subsequent mitochondrial disruption [17]. In type I cells, levels of caspase-8 activation are sufficient to directly activate the effector caspases, such as caspase-3. In type II cells, stimulation of the mitochondrial apoptotic pathway is essential for full effector caspases activation [18]. The inhibitor of apoptosis protein (IAPs) family comprises proteins that can bind and inactivate caspases. For example, X-linked IAP (XIAP) inhibits caspases-3 and -9 and its anti-

apoptotic activity is neutralized by the release of second mitochondria-derived activator of caspase (SMAC) from mitochondria [19]. More recently, death receptors have been discovered to trigger another way to die, named necroptosis. This caspase-independent form of regulated necrotic cell death has been mostly studied in TNF receptor signaling and appears important for the regulation of immunity and inflammation [20]. TNF-induced necroptosis depends on the activation of receptor interacting protein 1 (RIP1; also known as RIPK1), and RIP3 (also known as RIPK3), in a complex consisting of TRADD, FADD and caspase-8. Recently TRAIL was found to activate necroptosis in tumor cells under acidic extracellular pH conditions, involving RIP1 and RIP3 [21]. Whether TRAIL can also induce the formation of a similar, so-called 'necroptosome' complex, consisting of amongst others RIP1/RIP3 remains to be shown.

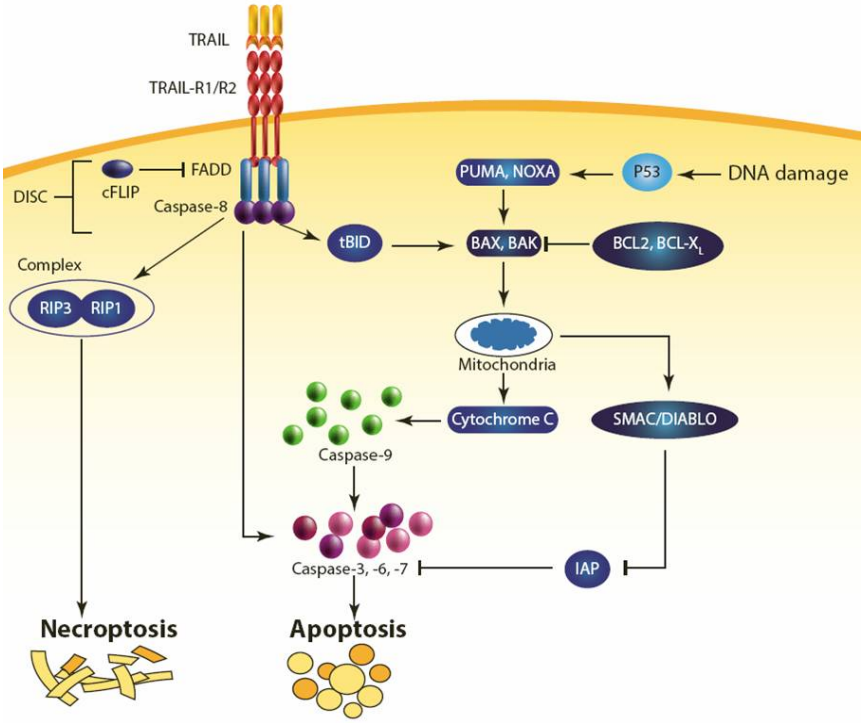

Figure 1. The TRAIL apoptotic pathway. Simplified and schematic representation of the pathway. Binding of TRAIL or generated agonistic agents to the TRAIL receptors results in the recruitment of FADD and procaspase-8, also known as the DISC. Subsequently, caspase-8 cleavage in the DISC can activate downstream caspase-3, leading to the induction of apoptosis (extrinsic pathway). The caspase-8 analogue cFLIP can compete for FADD binding and inhibit DISC formation. Caspase-8 can also cleave the Bcl-2 family member Bid into tBid engaging the intrinsic pathway by binding to Bax causing mitochondrial membrane permeabilization and the release of apoptogenic factors such as cytochrome c. Pro-survival Bcl2 family members can prevent mitochondrial permeabilization. IAPs, such as XIAP, can bind to caspases and inhibit apoptosis. Necroptosis, a regulated form of caspase-independent necrotic cell death, can also be activated through TRAIL receptors under specific conditions. This involves the activation of RIP1 and RIP3 in a complex that has not been precisely clarified as yet. See text for more details.

In preclinical studies, approximately half of the tumor cells show resistance towards TRAIL-induced apoptosis, but combined treatment with various standard or experimental agents can re-sensitize these cells (reviewed in [22;23]. Intrinsic TRAIL resistance in tumor cells involves blockades at different levels in the pathway, such as high levels of decoy receptors, limitations in DISC formation due to cFLIP-mediated inhibition, posttranslational modifications of DISC proteins, and high expression of anti-apoptotic Bcl-2 proteins that inhibit mitochondrial apoptosis. Combined therapy with standard chemo- and radiotherapy as well as a number of targeted agents can overcome these apoptotic blockades. Commonly these agents act by upregulation of TRAIL receptors and downregulation of anti-apoptotic proteins resulting in enhanced DISC formation and mitochondria-dependent apoptosis [24-32].

The promising antitumor activity in preclinical models has spurred early clinical studies with TRAIL receptor agonists in various tumor types [33-37]. However, as described below, recent preclinical findings indicate pro-survival, proliferation and even metastatic activity of TRAIL, suggesting more caution when applying these TRAIL receptor agonists, particularly as single agents. In this review we will give an update on the non-canonical (non-apoptotic) signaling activity of the TRAIL pathway in tumor and non-transformed cells. The different kinase cascades involved are described and the consequences for TRAIL-based therapy are discussed.

TRAIL RECEPTOR SIGNALING COMPLEXES AND KINASE ACTIVATION

The early molecular events leading to non-canonical TRAIL signaling are complex and not well understood. Co-immunoprecipitation experiments have indicated the formation of a so-called secondary signaling complex subsequent to the assembly of the primary DISC [38]. This secondary complex was found to contain RIP1, TNF receptor associated factor 2 (TRAF2) and NF-κB essential modulator (NEMO)/IKKγ in addition to FADD and active caspase-8. In this context, the localization of the TRAIL receptors in the cell membrane also appears to play a role in complex formation. In particular TRAIL receptor localization in lipid rafts enables apoptosis activation [39]. Lipid rafts are plasma membrane domains enriched in cholesterol and glycolsphingolipids function as signaling platforms that serve colocalization of requisite components. In the same study, TRAIL receptors complex assembly outside the rafts was associated with non-canonical signaling, mediated by RIP and cFLIP [39]. Aggregation of the TRAIL-R1 and -R2 in lipid rafts also occurred only in TRAIL sensitive non-small lung cancer (NSCLC) H460 cells and not in the resistant A549 cells [40]. Whether lipid raft localization of TRAIL receptors is a consequence or a cause of DISC formation remains to be proven.

Additional layers of regulation are provided by the expression of DISC inhibitors, such as cFLIP, and the phosphorylation and/or ubiquitination of several TRAIL receptor-interacting proteins, including caspase-8 and RIP1, which has been more extensively reviewed

elsewhere [16;41;42]. Regardless of the more precise composition and regulation of the complexes involved, non-canonical TRAIL receptor-induced kinase activation has been reported in various cell- and tumor types. These cascades were demonstrated to result in proinflammatory, proliferative, survival and migratory responses and are in more detail discussed below.

IκB/ NF-κB SURVIVAL SIGNALING

Activation of the inflammatory and cell survival pathway controlled by the transcription factor NF-κB was one of the first reported non-canonical signals elicited by TRAIL (see Figure 2 and Table 1). Both TRAIL-R1 and –R2 were found to activate NF-κB in a TRADD and RIP1-dependent way [43-45]. NEMO (IKK-γ) was found to be part of the secondary complex, able to recruit IKKα/β to the signaling complex causing the phosphorylation and subsequent proteasomal degradation of the inhibitor of kβ (IκB), leading to release and accumulation of NF-κB [38]. NF-κB then translocates to the nucleus where it can activate the transcription of amongst others the anti-apoptotic genes cFLIP, Bcl-x_L, Mcl-1 and cIAPs [46]. In preclinical tumor models such as (primary) leukemia [47], neuroblastoma [48], pancreatic cancer [49], mantle cell lymphoma cells [50] and NSCLC [51], inhibition by overexpression of an IκB dominant-negative version or by selective chemical inhibitors enhanced TRAIL apoptosis. Interestingly, NF-κB can have dual activity as it was found to mediate the upregulation of TRAIL receptors on one hand, and enhance Bcl-x_L expression and apoptosis resistance on the other [52]. More recently, a pro-apoptotic role for NF-κB was demonstrated in glioma cell lines where inhibition of the NF-κB pathway reduced TRAIL-induced apoptosis via an as yet elusive mechanism [53].

Table 1| Summary of the kinases activated by TRAIL in different tumor types. The functional consequences of activation being either pro-apoptotic, pro-survival, proliferative or migratory are indicated.

Kinase	Tumor type	Response	Reference
IκB/ NF-κB	leukemia, neuroblastoma, pancreatic, mantle cell lymphoma, NSCLC	pro-survival	[47-51]
	glioma	pro-apoptotic	[52]
JNK	lymphoid, cholangiocarcinoma	pro-apoptotic	[56;60;124]
	hepatocellular carcinoma	pro-survival	[63]
short JNK1	colon	pro-survival	[65]
long JNK1	colon	pro-apoptotic	[65]
p38	ovarian	pro-apoptotic	[66]
	prostate, breast	pro-survival	[68;69]
ERK	colon, SCLC, melanoma, glioma	proliferative	[71-74]
TAK1	prostate, ovarian	pro-survival	[68;77]
PKC	pancreatic	pro-survival	[79]
δ,ε,η	melanoma, breast	pro-survival	[80;81]
α,β,γ	NSCLC	pro-apoptotic	[24]
PI3K/Akt	leukaemia, ovarian, breast, NSCLC, prostate	pro-survival	[85;89-91]
Src	prostate, NSCLC, hepatic, breast	Migratory/invasive and pro-survival	[88;90;94;95]

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) IN TRAIL-SIGNALING

MAPKs are enzymes that control important physiological processes, such as gene expression, motility, metabolism, mitosis, and programmed cell death. In mammals six distinct groups of MAPKs have been characterized, extracellular regulated kinases (ERK1/2), Jun NH2 terminal kinases (JNK1/2/3), p38 (p38 α/β/γ/δ), ERK7/8, ERK3/4 and ERK5 [54]. TRAIL can activate JNK, p38 and ERK1/2 in several cancer cell lines with dual effects but mostly contributing to cell proliferation and pro-survival signalling, as outlined further below.

c-Jun N-terminal kinases (JNKs)

The JNKs are stress-activated members of the MAP kinase family that can be activated by TRAIL via both caspase-dependent and -independent mechanisms in a cell type-specific way [55]. FADD appeared to be dispensable for JNK activation. In lymphoid cells, JNK pathway activation by TRAIL contributed to apoptosis activation [56]. JNK activation involved a TRAF2-MEKK1-MKK4-dependent signaling pathway in human embryonic kidney 293 cells [57] and required RIP1 in prostate cancer cells [58]. Both TRAF2 and RIP1 were detected in the secondary complex and were required for JNK activation in fibrosarcoma cells [38]. JNK activation results in phosphorylation of its well-known target the transcription factor c-Jun/ AP1, but can also directly phosphorylate the pro-apoptotic Bcl-2 family member Bim in hepatocytes, thereby stabilizing the protein and facilitating mitochondrial apoptosis [59]. A role for JNK and Bim has also been found in the activation

of a lysosmal death pathway induced by TRAIL in cholangiocarcinoma cells [60]. Another cell death route was also connected to TRAIL receptor-dependent activation of JNK; JNK appeared to phosphorylate a key autophagy regulator, Beclin-1, leading to autophagic cell death in HCT116 cells [61]. Synergistic apoptosis activation by chemotherapy and TRAIL receptor agonists was shown to involve MKK4-dependent JNK activation. MAPK p38 was also activated and suppression of activation of these kinases by the antioxidant N-acetyl cysteine prevented synergistic effects [62]. However, in contrast, direct inhibition of JNK by RNA interference or chemical inhibition augmented TRAIL-induced apoptosis in hepatocellular carcinoma cells [63]. This indicates that JNK can have opposite effects in TRAIL signaling that may be dependent on the cellular context. These dual functions can in part be due to the duration or magnitude of the activation of the pathway. For example, it was found that prolonged activation of JNK by TNF induces apoptosis, whereas transient activation of JNK promotes cell survival [64]. Furthermore, a recent study has shown that in colon cancer cell lines the short JNK1 isoforms (JNK1α1 and JNK1β1) transmit an anti-apoptotic signal, whereas the long isoforms (JNK1α2 and JNK1β2) are pro-apoptotic upon activation of TRAIL [65]. This may also provide an explanation for the dual role of JNK.

P38 MAPK

Several reports have shown that TRAIL can activate p38 MAPK. P38 activation can occur through the formation of the secondary complex consisting of FADD, caspase-8, RIP1 and TRAF2 [38]. Lee and co-workers found TRAIL-induced p38 activation in Hela cells to be responsible for caspase activation and apoptosis. Elevation of reactive oxygen species (ROS) by TRAIL appeared instrumental for p38 activation [66]. P38 activation by TRAIL was also observed in DLD1 colon cancer cells however; in these cells inhibition of p38 did not affect TRAIL-mediated cell death [67]. In yet other tumor cells p38 was found to suppress apoptosis. In prostate cancer cells, TRAIL induced p38 phosphorylation causing the transcriptional upregulation of anti-apoptotic Bcl-2 family member Mcl-1, thus rescuing the prostate cancer cells from apoptosis. Inhibition of p38 with chemical inhibitor, SB203580, increased the level of cell death by TRAIL in these cells [68]. Breast carcinoma cells could also be sensitized for TRAIL after inhibition of p38 indicating that this kinase contributes to cell survival in these cells [69]. Thus, p38 can either suppress or enhance the apoptotic effect of TRAIL in a cell type specific way.

Extracellular-signal-regulated kinases (ERKs)

The activation of ERKs by TRAIL has also been reported in a number of reports. In neuroblastoma cells, TRAIL-induced ERK1/2 phosphorylation [70], and the inhibition of ERK1/2 could enhance TRAIL-dependent death in colon cancer cells [71]. Even more, in small cell lung cancer (SCLC) cells lacking caspase-8, TRAIL caused cell proliferation, which was TRAIL-R2 mediated in some but not all SCLC cell lines tested [72]. TRAIL-induced

proliferation could be prevented by chemical inhibition or siRNA-mediated knockdown of ERK1/2 identifying this pathway as a critical proliferation mediator [72]. Also in a panel of human melanoma cell lines with variable sensitivities for TRAIL, ERK1/2 phosphorylation were detected within 30 minutes of TRAIL treatment [73]. ERK1/2 inhibition resulted in downregulation of Bcl-2, Bcl-x_L and Mcl-1 expression providing an explanation for enhanced TRAIL-induced mitochondrial apoptosis in these cells. TRAIL resistant human glioma cells also demonstrated enhanced cell proliferation upon TRAIL treatment that could be linked to ERK1/2 activation [74]. ERK inhibition suppressed stimulation of proliferation but did not sensitize for TRAIL. Knockdown of cFLIP on the other hand prevented ERK activation and resulted in partial sensitization for TRAIL-dependent apoptosis [74]. Thus, the activation of ERK by TRAIL generally has been implicated in stimulation of cell survival and proliferation of tumor cells.

As mentioned, the secondary complex and active caspase-8 have been implicated in MAPK activation [38]. However, an alternative caspase-8 requiring mechanism was reported by Song and Lee in DU-145 prostate cancer cells [75]. They found that mammalian sterile 20-like kinase 1 (Mst1), a ubiquitously expressed serine/threonine kinase and caspase substrate, could activate p38 and JNK through a caspase-7 cleavage generated 40 kDa form of Mst1. A caspase-3 generated 36 kDa form of Mst1 could activate ERK [75]. The same group reported MAP3K (MEKK1) and stress-activated protein/ERK kinase 1 (SEK1) to mediate TRAIL-induced JNK/p38 phosphorylation also requiring active caspase-8 [76].

TGF-β-activated kinase 1 (TAK1)

TAK1 is a member of the MAP3K family and is activated by various cytokines, such as the TGF-β, TNF-α, interleukin-1 (IL-1), and ligands of the Toll-like receptors. It is a key regulator of the NF-κB subunit p65/RelA and MAPKs activity. TRAIL activated TAK1 in Hela cells resulting in p65, JNK and p38 activation; siRNA knockdown or chemical inhibition of TAK1 enhanced TRAIL-induced apoptosis [77]. The earlier mentioned activation of p38 by TRAIL in prostate cancer cells was preceded and dependent on activation of TAK1 leading to transcriptional upregulation of Mcl-1 and suppression of apoptosis [68]. Inhibition of TAK1 is an effective approach to increase TRAIL sensitivity as was reported by Morioka and co-workers, although in their study direct phosphorylation of TAK1 after TRAIL was not demonstrated [78]. In fibroblasts and keratinocytes derived from TAK-/- knockout mice, as well as in tumor cell lines Saos2 and HeLa cells with silenced TAK1 expression, sensitization to TRAIL-killing was observed independent of NF-κB activity. In the absence of TAK1 TRAIL exposure resulted in ROS accumulation and subsequent degradation of cIAP leading to caspase-3 activation and apoptosis. Overall, TAK1 activation by TRAIL appears to be associated with apoptosis resistance through either affecting NF-κB signaling and/ or JNK, p38 activation.

DUAL EFFECTS ON APOPTOSIS BY PROTEIN KINASE C (PKC)

A number of studies have reported on the TRAIL receptor-dependent activation of protein kinase C (PKC) and its isoforms. Activation of PKC by TRAIL in resistant pancreatic adenocarcinoma cells had anti-apoptotic effects, and PKC inhibition with Gö6983 sensitized for apoptosis [79]. Furthermore, TRAIL-sensitive cells could be made resistant following PKC activation with phorbol 12-myristate 13-acetate (PMA). In apoptosis sensitive melanoma cells, TRAIL stimulated the phosphorylation of PKCδ and PKCε resulting in apoptosis protecting effects [80]. In the same study, activation of PKC by PMA suppressed apoptosis by preventing the translocation and activation of pro-apoptotic Bax to the mitochondria and subsequent apoptosis. Other studies also reported extracellular stimuli-dependent activation of PKC, in particular of the isotypes PKCε and PKCη. For example, in breast cancer cells, PKCε caused TRAIL resistance by activating Akt, followed by Hdm2 phosphorylation. Hdm2, on its turn reduced p53 expression, leading to downregulation of Bid and suppression of activation of the mitochondrial pathway [81]. Activation of PKC also inhibited the recruitment of FADD and caspase-8 resulting in disruption of DISC formation and a decrease in apoptosis [82].

In contrast to the mentioned anti-apoptotic effects of TRAIL- and extracellular-induced PKC activation, the activation of conventional PKC isoforms (α, β, γ) by PMA or bryostatin-1 was reported to increase the expression of pro-apoptotic Bad and the TRAIL receptors leading to sensitization for apoptosis by TRAIL [83]. In NSCLC, PKCα and PKCβ increased TRAIL-R2 expression and sensitized for TRAIL-induced apoptosis [24]. Thus, it appears that activation of PKCδ, PKCε and PKCη leads to apoptosis inhibition, whereas activation of PKC isoforms α, β and γ enhances apoptosis induced by TRAIL which is cell type dependent.

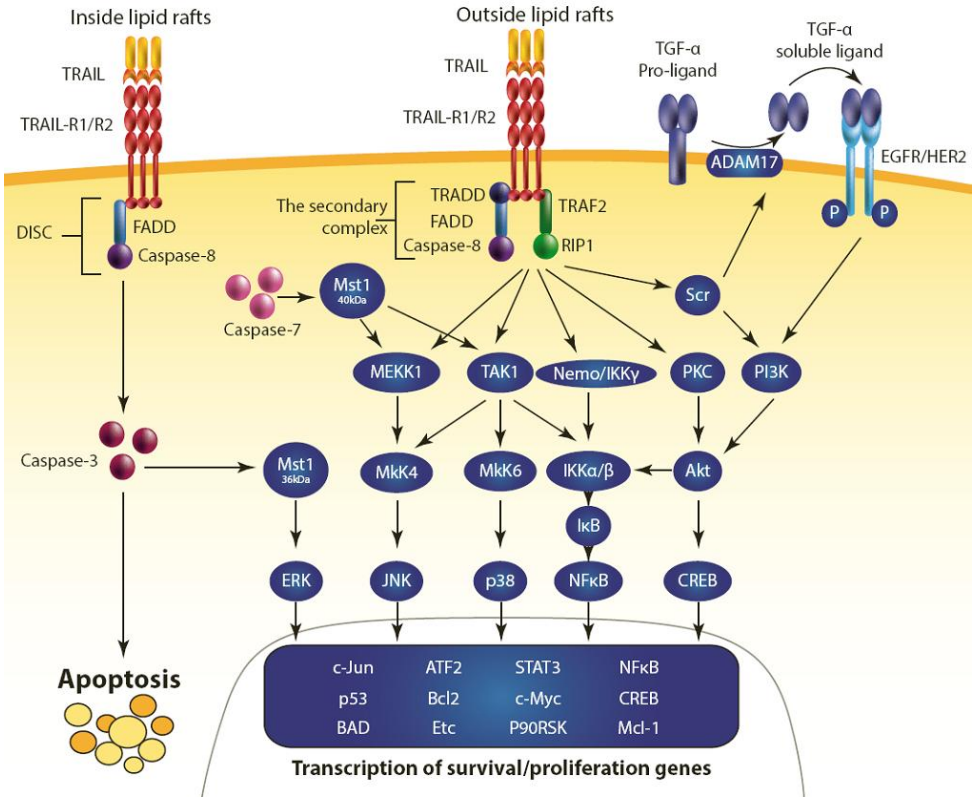

Figure 2. Canonical and non-canonical TRAIL signalling in cancer cells. Schematic overview of apoptotic, and proliferation/ pro-survival signals elicited by the activation of TRAIL receptors. Following binding of TRAIL receptor agonists to their death receptors, the DISC can be formed, resulting in apoptosis. A secondary complex can also be formed after TRAIL receptor activation leading to the activation of various kinases and the induction of direct or indirect non-apoptotic responses as indicated. The more precise molecular events required for secondary complex formation are subject of investigations. The complex could be associated with TRAIL receptors at the membrane or formed in the cytoplasm. See text for more details.

PHOSPHATIDYLINOSITIDE 3-KINASES (PI3K)/AKT SIGNALING COUNTERACTS APOPTOSIS

Akt or protein kinase B (PKB) is one of the most critical kinases in the regulation of cell survival. Enhanced activity of the PI3K/Akt pathway is found in many malignancies and is associated with the stimulation of cell growth and cell survival [84]. In leukemic T Jurkat cells, TRAIL phosphorylated PI3K and Akt within 30 minutes, and inhibition of PI3K with the pharmacological inhibitor LY294002 sensitized cells for TRAIL-induced apoptosis [85]. Sensitization was associated with reduced nuclear translocation of NF-κB p65, reflecting an earlier found direct ability of PI3K/Akt to phosphorylate and transactivate p65 and NF-κB signaling upon TNF treatment [86;87]. However, NF-κB inhibition did not sensitize for

TRAIL apoptosis but enhanced necrotic cell death. More recently, cross-talk between Akt and NF-κB was found to involve direct phosphorylation of IKKβ by Akt leading to nuclear translocation of NF-κB and increased anti-apoptotic gene expression causing resistance for TRAIL [88].

TRAIL-induced Akt phosphorylation has been reported in various other tumor cells. In TRAIL resistant ovarian and breast cancer cell lines, TRAIL exposure induced phosphorylation of Akt and its substrate the serine/threonine kinase mTOR (mammalian target of rapamycin) within hours of treatment [89]. The cells could be sensitized for TRAIL by adding the PI3K inhibitor LY294002 or by restoring the expression of the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (PTEN) in PTEN negative tumor cells. PTEN inhibits the PI3K/Akt pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate (PIP3), a cofactor for Akt activation. Recently, also TRAIL-dependent phosphorylation of Akt was reported in TRAIL resistant NSCLC cells and inhibition of Akt resulted in sensitization for TRAIL apoptosis [90]. Akt activation has also been described in TRAIL-sensitive prostate adenocarcinoma DU-145 cells [91]. Akt inhibition enhanced apoptosis activation by TRAIL and interestingly, roles for the Rous sarcoma oncogene cellular homolog (Src), and Casitas B-lineage lymphoma (c-Cbl) kinases were found upstream of Akt in this signaling route. Based on work in the same prostate cancer cell line, Song and coworkers proposed TRAIL-induced Akt activation to be in part responsible for the development of acquired TRAIL resistance [92]. In this model, Akt phosphorylates its substrate Bad, another Bcl-2 family protein, leading to inactivation of the pro-apoptotic function of this BH3-only protein and suppression of mitochondrial apoptosis. Moreover, the same group identified another TRAIL-dependent route leading to Akt activation, involving a cascade of p38 and subsequent HSP27 phosphorylation that was required for catalytic Akt activation [76]. An interesting but mechanistically poorly understood phenomenon is the recent finding that ectopic expression of TRAIL-R4/ DcR2 in Hela cells enhanced cell growth in vitro and as xenograft transplants in mice in a ligand-independent way [93]. In these cells constitutive Akt activation was detected and inhibition of Akt suppressed tumor growth. Overall, the activation of the PI3K/Akt pathway by TRAIL has been demonstrated in several tumor models showing that inhibition of this route sensitizes for apoptosis.

DIRECT AND INDIRECT ACTIVATION OF SRC SUPPRESSES APOPTOSIS

As mentioned earlier, in DU-145 prostate cancer cells TRAIL-induced Akt activation was mediated by the non-receptor tyrosine kinase Src and inhibition of Src sensitized cells for apoptosis [91]. In line with this, the inhibition of Src could restore TRAIL sensitivity in resistant hepatic carcinoma cells by facilitating caspase-8 cleavage [94]. Interesting in this respect is the finding that survival of metastatic breast cancer cells in the bone of mice was dependent on Src [95]. Intriguingly, Src activation amongst other pathways was

associated with TRAIL resistance allowing the survival of disseminated breast cancer cells in the bone marrow by rendering them resistant to TRAIL produced by the microenvironment. Mechanistically, Src has been directly linked with apoptosis resistance. The epidermal growth factor (EGF) was found to promote Src-mediated phosphorylation of caspase-8 at Tyrosine 380 thus impairing death receptor/ caspase-8-dependent apoptosis [96]. Another link between TRAIL-induced Src activation and resistance to apoptosis has been identified that involves the indirect activation of the EGF receptor (EGFR) [97]. TRAIL could stimulate signaling by human epidermal receptor (HER) family members EGFR and HER2 in colorectal cancer cells by activating the Src family kinases (SFK), which in turn activates a disintegrin and metalloproteinase (ADAM) family member ADAM-17 leading to the shedding of TGF-α. Subsequently, TGF-α, a ligand of EGFR, activated the EGFR/HER2 pro-survival signaling in an autocrine and paracrine manner [97]. Taken together, Src activation has been consistently associated with TRAIL signaling and apoptosis resistance.

MECHANISMS OF TRAIL-INDUCED MIGRATION AND INVASION

When considering the physiological function of TRAIL, which is thought to include innate immune surveillance against tumor development by suppressing tumor initiation and metastasis [98;99], the more recently found pro-invasive effects of TRAIL on tumor cells were surprising (see also Figure 3). Ishimura et al. reported that TRAIL administration stimulates cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells in a NF-κB dependent manner [100]. In another study, the administration of aggregated TRAIL enhanced primary tumor growth and also stimulated the formation of distant metastases in an orthotopic xeno-transplantation model of human pancreatic ductal adenocarcinoma cells overexpressing Bcl-x_L (Colo357/ Bcl-x_L) [101]. In these cells, TRAIL stimulated the expression of proinflammatory cytokines, such as interleukin-8 (IL-8), and urokinase-type plasminogen activator (uPA), which were linked to invasive behaviour [101]. The activation of matrix metalloproteinase-9 (MMP9), which is an important angiogenic and prognostic factor in malignant tumors, by TRAIL has also been reported in NSCLC, pancreatic cancer, and human malignant astrocytoma cells [102-104]. In colon cancer HCT116 cells, ectopic expression of mutated PI3K catalytic subunit alpha (PIK3CA) blocked TRAIL-induced apoptosis downstream of caspase-8 cleavage leading to induction of proinflammation and cell invasion responses [105]. The oncogenic proteins, K-RAS and Raf-1 were mentioned as critical factors in switching the pro-apoptotic function of death receptors into a pro-invasive function in these colon cancer cells. Evidence was provided that these oncogenic kinases convert death receptors into invasion-inducing receptors by suppressing the Rho kinase (ROCK)/LIM kinase-mediated phosphorylation of the actin-severing protein cofilin that is a regulator of actin dynamics during cell invasion [106]. However, whether the suggested critical role of K-RAS and RAF-1 in switching the function

of death receptors to invasive receptors is of more general relevance will need further confirmation in a broader panel of tumor cells. More recently, the RIP1/Src/STAT3 axis was identified to mediate TRAIL-dependent migration and invasion of TRAIL resistant NSCLC cells [90]. Using TRAIL receptor selective TRAIL variants, TRAIL-R2 was found to be the main mediator of invasion. In this model, inhibition of Src as well as STAT3 prevented invasion but did not notably sensitize for TRAIL. Overall, it appears that tumor cells have altered the anti-metastatic activity of TRAIL into an invasion stimulatory signal thus using this death pathway to their malignant benefit.

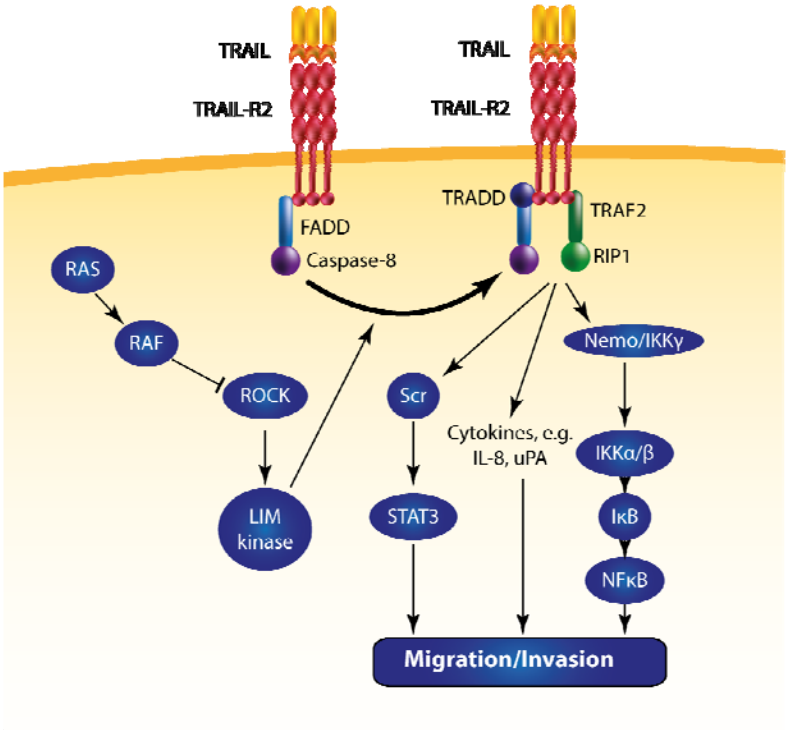

Figure 3. TRAIL-dependent routes that can induce migration and invasion of tumor cells. Schematic representations of the mechanism identified that mediate TRAIL-induced tumor cell invasion. The composition of the different protein complexes induced by TRAIL and the mechanisms controlling this remain to be further elucidated. See text for more details.

NON-APOPTOTIC TRAIL SIGNALING IN NON-TRANSFORMED CELLS

Non-canonical kinase activation by TRAIL is also known to occur in normal healthy tissue cells that are resistant to the killing effect of TRAIL receptor agonists [107]. TRAIL-dependent proinflammatory, pro-survival, proliferation and cell migratory responses have been observed in different cell types. For example, TRAIL activated Akt-dependent survival and ERK1/2-dependent proliferation in human endothelial cells (ECs) [108]. In EC cells TRAIL was also reported to induce NF-κB signaling leading to enhanced expression of

adhesion molecules [109]. Furthermore, ECs exposed to TRAIL displayed enhanced migration and vessel tube formation ability suggestive of pro-angiogenic activity of TRAIL [110]. However, a recent study by Wilson et al. showed that activation of murine TRAIL-R disrupts tumor – and not normal vasculature in tumor bearing mice models suggesting differences in TRAIL signaling networks between normal and tumor stromal ECs [111]. In vascular smooth muscle cells (VSMCs), TRAIL stimulated the production of the proinflammatory cytokines TNFα, IL-1β and INFγ leading to apoptosis protection and induction of proliferation and migration in an ERK1/2-dependent manner [112]. In addition, several other mechanisms have been reported to contribute to these effects of TRAIL, such as activation of NF-κB, insulin-like growth factor type 1 receptor (IGF1R) and SP-1 [113;114]. Furthermore, TRAIL could stimulate p38-dependent INFγ secretion and proliferation in T cells [115], enhance survival signaling in eosinophils of astma patients [116], and stimulate ERK1/2 and Akt-dependent survival of fibroblast-like synoviocytes derived from rheumatoid arthritis patients [117].

Interestingly, also a role for TRAIL signaling has been found in the differentiation of different cell types. In intestinal mucosa cells, TRAIL acted as a growth arrest mediator via activation of cyclin-dependent kinases p21 and p27 thought to stabilize the differentiated phenotype [118]. In human keratinocytes, TRAIL induced both caspase-dependent differentiation and apoptosis [119]. Furthermore, TRAIL induced differentiation of macrophage lineage precursors into osteoclasts by stimulating proinflammatory cytokine production via NF-κB, ERK and p38 activation and independent from caspase cleavage [120]. The molecular mechanisms causing the activation of these alternative TRAIL signaling routes in normal cells remain largely elusive. Some light on this was shed recently by the observation that in mouse embryonal fibroblasts derived of TAK1 and TRADD knock-out mice apoptosis sensitivity to TRAIL was detected. TRADD and TAK1 could be linked to the activation of NF-κB-dependent pro-survival signals, such as upregulation of cFLIP thus causing resistance [121;122]. Another study proposed that recruitment of TRADD to the TRAIL receptor reduces the levels of FADD in the complex while stimulating RIP1 interactions leading to pro-survival signals [123]. Thus, it appears that similar non-canonical signals can be activated by TRAIL in non-transformed normal cells as in TRAIL-resistant tumor cells.

CONCLUDING REMARKS

In pursue of novel potent tumor-selective apoptosis-inducing strategies the TRAIL receptors provide excellent targets on first sight. Indeed, their favourable property of triggering tumor-selective apoptosis has been demonstrated in many preclinical studies. However, it is becoming clear that signaling via the TRAIL receptors is much more complex than initially thought and can have different functional outcomes as is illustrated here. TRAIL receptor activation can have antitumor effects by inducing caspase-dependent apoptosis in TRAIL sensitive cells, whereas in TRAIL resistant tumor cells, the activation can lead to protumorigenic effects such as enhanced proliferation, survival and invasion. Whether TRAIL-induced necroptosis contributes to anti-tumor activity remains to be demonstrated. The effects of TRAIL receptor activation in resistant tumor cells resemble that observed in non-transformed apoptosis resistant (normal) cells. This suggests that the protumorigenic mechanisms elicited by TRAIL in resistant tumor cells are actually part of normal physiological signaling in non-transformed cells. However, an important difference is that combination therapies that sensitize cancer cells for TRAIL-induced apoptosis are in most cases not harmful for normal cells, indicating specific changes in tumor cells allowing the activation of caspase-dependent apoptosis.

The precise kinase cascades triggered by TRAIL remain to be further identified, which is complicated due to cross-activation between kinases, cell type-dependent variation and the effects of external stimuli produced by the tumor microenvironment. A number of studies have attempted to unravel the molecular mechanisms responsible for the dichotomy in TRAIL signaling but the underlying mechanisms remain poorly understood. Thus far the DISC (FADD, caspase-8) and the signaling complex (FADD, , caspase-8, RIP1, TRAF2, NEMO) have been identified [38] as main mediators of apoptosis and non-apoptotic signals, respectively. RIP1 dependent signalling can stimulate cell survival and even tumor cell invasion. In addition, TRAIL has been shown to induce RIP1-dependent necroptosis under specific conditions. The more precise mechanisms by which RIP1 can transmit these signals remain to be determined. Clearly more research is required to unravel the molecular mechanism regulating non-canonical TRAIL and to identify molecular switches that may be used for setting the system into the ‘apoptosis position’. This will provide new clues for developing better strategies for using TRAIL receptor agonists for the treatment of cancer.

Acknowledgements

This work was performed within the framework of project T3-112 of the Dutch Top Institute Pharma and supported by grant RUG2011-5211 from the Dutch Cancer Society. We thank Esther van Straten for making the figures.

Reference List

1. Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov. 2008; 7(12):1001-1012.
2. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996; 271(22):12687-12690.
3. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995; 3(6):673-682.
4. Fulda S, Debatin KM. Exploiting death receptor signaling pathways for tumor therapy. Biochim Biophys Acta 2004; 1705(1):27-41.
5. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003; 10(1):66-75.
6. Kim K, Fisher MJ, Xu SQ, El-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 2000; 6(2):335-346.
7. Nimmanapalli R, Perkins CL, Orlando M, O'Bryan E, Nguyen D, Bhalla KN. Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. Cancer Res 2001; 61(2):759-763.
8. Petak I, Tillman DM, Harwood FG, Mihalik R, Houghton JA. Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. Cancer Res 2000; 60(10):2643-2650.
9. Reis CR, van der Sloot AM, Szegezdi E, Natoni A, Tur V, Cool RH et al. Enhancement of antitumor properties of rhTRAIL by affinity increase toward its death receptors. Biochemistry 2009; 48(10):2180-2191.
10. Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A et al. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. J Biol Chem 2008; 283(29):20560-20568.
11. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. Proc Natl Acad Sci U S A 2006; 103(23):8634-8639.
12. Lemke J, Noack A, Adam D, Tchikov V, Bertsch U, Roder C et al. TRAIL signaling is mediated by DR4 in pancreatic tumor cells despite the expression of functional DR5. J Mol Med (Berl) 2010; 88(7):729-740.
13. MacFarlane M, Inoue S, Kohlhaas SL, Majid A, Harper N, Kennedy DB et al. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. Cell Death Differ 2005; 12(7):773-782.
14. Bellail AC, Tse MC, Song JH, Phuphanich S, Olson JJ, Sun SY et al. DR5-mediated DISC controls caspase-8 cleavage and initiation of apoptosis in human glioblastomas. J Cell Mol Med 2010; 14(6A):1303-1317.
15. Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, Deforge L et al. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. J Biol Chem 2005; 280(3):2205-2212.
16. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. Biochim Biophys Acta 2010; 1805(2):123-140.
17. Ozoren N, El-Deiry WS. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. Neoplasia 2002; 4(6):551-557.
18. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nat Rev Immunol 2007; 7(7):532-542.

19. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102(1):33-42.
20. Vanlangenakker N, Vanden Berghe T, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 2012; 19(1):75-86.
21. Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van HF et al. TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. Cell Death Differ 2012.
22. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39(7-8):1462-1475.
23. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
24. Chen W, Wang X, Zhuang J, Zhang L, Lin Y. Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. Carcinogenesis 2007; 28(10):2114-2121.
25. Duiker EW, Meijer A, van der Bilt AR, Meersma GJ, Kooi N, van der Zee AG et al. Drug-induced caspase 8 upregulation sensitises cisplatin-resistant ovarian carcinoma cells to rhTRAIL-induced apoptosis. Br J Cancer 2011; 104(8):1278-1287.
26. Elrod HA, Lin YD, Yue P, Wang X, Lonial S, Khuri FR et al. The alkylphospholipid perifosine induces apoptosis of human lung cancer cells requiring inhibition of Akt and activation of the extrinsic apoptotic pathway. Mol Cancer Ther 2007; 6(7):2029-2038.
27. Frese S, Brunner T, Gugger M, Uduehi A, Schmid RA. Enhancement of Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in non-small cell lung cancer cell lines by chemotherapeutic agents without correlation to the expression level of cellular protease caspase-8 inhibitory protein. J Thorac Cardiovasc Surg 2002; 123(1):168-174.
28. Hellwig CT, Rehm M. TRAIL signaling and synergy mechanisms used in TRAIL-based combination therapies. Mol Cancer Ther 2012; 11(1):3-13.
29. Siegelin MD, Habel A, Gaiser T. 17-AAG sensitized malignant glioma cells to death-receptor mediated apoptosis. Neurobiol Dis 2009; 33(2):243-249.
30. Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB. Gossypol induces death receptor-5 through activation of the ROS-ERK-CHOP pathway and sensitizes colon cancer cells to TRAIL. J Biol Chem 2010; 285(46):35418-35427.
31. Wang S, Ren W, Liu J, Lahat G, Torres K, Lopez G et al. TRAIL and doxorubicin combination induces proapoptotic and antiangiogenic effects in soft tissue sarcoma in vivo. Clin Cancer Res 2010; 16(9):2591-2604.
32. Zhang X, Cheung RM, Komaki R, Fang B, Chang JY. Radiotherapy sensitization by tumor-specific TRAIL gene targeting improves survival of mice bearing human non-small cell lung cancer. Clin Cancer Res 2005; 11(18):6657-6668.
33. Hotte SJ, Hirte HW, Chen EX, Siu LL, Le LH, Corey A et al. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. Clin Cancer Res 2008; 14(11):3450-3455.
34. Mahalingam D, Oldenhuis CN, Szegezdi E, Giles FJ, de Vries EG, de Jong S et al. Targeting TRAIL towards the clinic. Curr Drug Targets 2011; 12(14):2079-2090.
35. Merchant MS, Geller JI, Baird K, Chou AJ, Galli S, Charles A et al. Phase I trial and pharmacokinetic study of lexatumumab in pediatric patients with solid tumors. J Clin Oncol 2012; 30(33):4141-4147.
36. Soria JC, Smit E, Khayat D, Besse B, Yang X, Hsu CP et al. Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer. J Clin Oncol 2010; 28(9):1527-1533.

37. Tolcher AW, Mita M, Meropol NJ, von MM, Patnaik A, Padavic K et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007; 25(11):1390-1395.
38. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
39. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM et al. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. Cancer Res 2007; 67(14):6946-6955.
40. Ouyang W, Yang C, Liu Y, Xiong J, Zhang J, Zhong Y et al. Redistribution of DR4 and DR5 in lipid rafts accounts for the sensitivity to TRAIL in NSCLC cells. Int J Oncol 2011; 39(6):1577-1586.
41. Vucic D, Dixit VM, Wertz IE. Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol 2011; 12(7):439-452.
42. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009; 10(4):348-355.
43. Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. Immunity 1997; 7(6):821-830.
44. Schneider P, Thome M, Burns K, Bodmer JL, Hofmann K, Kataoka T et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity 1997; 7(6):831-836.
45. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997; 277(5327):818-821.
46. Kruyt FA. TRAIL and cancer therapy. Cancer Lett 2008; 263(1):14-25.
47. Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. Oncogene 2003; 22(25):3842-3852.
48. Karacay B, Sanlioglu S, Griffith TS, Sandler A, Bonthius DJ. Inhibition of the NF-kappaB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells. Cancer Gene Ther 2004; 11(10):681-690.
49. Braeuer SJ, Buneker C, Mohr A, Zwacka RM. Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells. Mol Cancer Res 2006; 4(10):715-728.
50. Roue G, Perez-Galan P, Lopez-Guerra M, Villamor N, Campo E, Colomer D. Selective inhibition of IkappaB kinase sensitizes mantle cell lymphoma B cells to TRAIL by decreasing cellular FLIP level. J Immunol 2007; 178(3):1923-1930.
51. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 2007; 6(7):2103-2112.
52. Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gelinas C et al. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. Nat Cell Biol 2001; 3(4):409-416.
53. Jennewein C, Karl S, Baumann B, Micheau O, Debatin KM, Fulda S. Identification of a novel pro-apoptotic role of NF-kappaB in the regulation of T. Oncogene 2012; 31(11):1468-1474.
54. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001; 410(6824):37-40.
55. Muhlenbeck F, Haas E, Schwenzer R, Schubert G, Grell M, Smith C et al. TRAIL/Apo2L

activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. J Biol Chem 1998; 273(49):33091-33098.
56. Herr I, Wilhelm D, Meyer E, Jeremias I, Angel P, Debatin KM. JNK/SAPK activity contributes to TRAIL-induced apoptosis. Cell Death Differ 1999; 6(2):130-135.
57. Hu WH, Johnson H, Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. J Biol Chem 1999; 274(43):30603-30610.
58. Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol 2000; 20(18):6638-6645.
59. Corazza N, Jakob S, Schaer C, Frese S, Keogh A, Stroka D et al. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. J Clin Invest 2006; 116(9):2493-2499.
60. Werneburg NW, Guicciardi ME, Bronk SF, Kaufmann SH, Gores GJ. Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. J Biol Chem 2007; 282(39):28960-28970.
61. Park KJ, Lee SH, Lee CH, Jang JY, Chung J, Kwon MH et al. Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem Biophys Res Commun 2009; 382(4):726-729.
62. Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T. Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. Oncogene 2003; 22(13):2034-2044.
63. Mucha SR, Rizzani A, Gerbes AL, Camaj P, Thasler WE, Bruns CJ et al. JNK inhibition sensitises hepatocellular carcinoma cells but not normal hepatocytes to the TNF-related apoptosis-inducing ligand. Gut 2009; 58(5):688-698.
64. Ventura JJ, Hubner A, Zhang C, Flavell RA, Shokat KM, Davis RJ. Chemical genetic analysis of the time course of signal transduction by JNK. Mol Cell 2006; 21(5):701-710.
65. Mahalingam D, Keane M, Pirianov G, Mehmet H, Samali A, Szegezdi E. Differential activation of JNK1 isoforms by TRAIL receptors modulate apoptosis of colon cancer cell lines. Br J Cancer 2009; 100(9):1415-1424.
66. Lee MW, Park SC, Yang YG, Yim SO, Chae HS, Bach JH et al. The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. FEBS Lett 2002; 512(1-3):313-318.
67. Zhang L, Zhu H, Davis JJ, Jacob D, Wu S, Teraishi F et al. Lack of p38 MAP kinase activation in TRAIL-resistant cells is not related to the resistance to TRAIL-mediated cell death. Cancer Biol Ther 2004; 3(3):296-301.
68. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of MCL-1. Cell Death Differ 2010; 17(8):1288-1301.
69. Weldon CB, Parker AP, Patten D, Elliott S, Tang Y, Frigo DE et al. Sensitization of apoptotically-resistant breast carcinoma cells to TNF and TRAIL by inhibition of p38 mitogen-activated protein kinase signaling. Int J Oncol 2004; 24(6):1473-1480.
70. Milani D, Zauli G, Rimondi E, Celeghini C, Marmiroli S, Narducci P et al. Tumour necrosis factor-related apoptosis-inducing ligand sequentially activates pro-survival and pro-apoptotic pathways in SK-N-MC neuronal cells. J Neurochem 2003; 86(1):126-135.
71. Vaculova A, Hofmanova J, Soucek K, Kozubik A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. FEBS Lett 2006; 580(28-29):6565-6569.
72. Belyanskaya LL, Ziogas A, Hopkins-Donaldson S, Kurtz S, Simon HU, Stahel R et al. TRAIL-induced survival and proliferation of SCLC cells is mediated by ERK and dependent on TRAIL-R2/DR5 expression in the absence of caspase-8. Lung Cancer 2008; 60(3):355-365.
73. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects

melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene 2003; 22(19):2869-2881.
74. Vilimanovich U, Bumbasirevic V. TRAIL induces proliferation of human glioma cells by c-FLIPL-mediated activation of ERK1/2. Cell Mol Life Sci 2008; 65(5):814-826.
75. Song JJ, Lee YJ. Differential cleavage of Mst1 by caspase-7/-3 is responsible for TRAIL-induced activation of the MAPK superfamily. Cell Signal 2008; 20(5):892-906.
76. Sun BK, Kim JH, Nguyen HN, Kim SY, Oh S, Lee YJ et al. TRAIL-induced caspase/p38 activation is responsible for the increased catalytic and invasive activities of Akt. Int J Oncol 2011; 38(1):249-256.
77. Choo MK, Kawasaki N, Singhirunnusorn P, Koizumi K, Sato S, Akira S et al. Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade. Mol Cancer Ther 2006; 5(12):2970-2976.
78. Morioka S, Omori E, Kajino T, Kajino-Sakamoto R, Matsumoto K, Ninomiya-Tsuji J. TAK1 kinase determines TRAIL sensitivity by modulating reactive oxygen species and cIAP. Oncogene 2009; 28(23):2257-2265.
79. Trauzold A, Wermann H, Arlt A, Schutze S, Schafer H, Oestern S et al. CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-kappaB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. Oncogene 2001; 20(31):4258-4269.
80. Gillespie S, Zhang XD, Hersey P. Variable expression of protein kinase C epsilon in human melanoma cells regulates sensitivity to TRAIL-induced apoptosis. Mol Cancer Ther 2005; 4(4):668-676.
81. Shankar E, Sivaprasad U, Basu A. Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53. Oncogene 2008; 27(28):3957-3966.
82. Harper N, Hughes MA, Farrow SN, Cohen GM, MacFarlane M. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. J Biol Chem 2003; 278(45):44338-44347.
83. Farrow B, Thomas RP, Wang XF, Evers BM. Activation of conventional PKC isoforms increases expression of the pro-apoptotic protein Bad and TRAIL receptors. Int J Gastrointest Cancer 2002; 32(2-3):63-72.
84. Fresno Vara JA, Casado E, de CJ, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 2004; 30(2):193-204.
85. Zauli G, Sancilio S, Cataldi A, Sabatini N, Bosco D, Di PR. PI-3K/Akt and NF-kappaB/IkappaBalpha pathways are activated in Jurkat T cells in response to TRAIL treatment. J Cell Physiol 2005; 202(3):900-911.
86. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999; 401(6748):82-85.
87. Wang D, Baldwin AS, Jr. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. J Biol Chem 1998; 273(45):29411-29416.
88. Tanaka H, Fujita N, Tsuruo T. 3-Phosphoinositide-dependent protein kinase-1-mediated IkappaB kinase beta (IkkB) phosphorylation activates NF-kappaB signaling. J Biol Chem 2005; 280(49):40965-40973.
89. Xu J, Zhou JY, Wei WZ, Wu GS. Activation of the Akt survival pathway contributes to TRAIL resistance in cancer cells. PLoS One 2010; 5(4):e10226.
90. Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. J Cell Sci 2012.
91. Song JJ, Kim JH, Sun BK, Alcala MA, Jr., Bartlett DL, Lee YJ. c-Cbl acts as a mediator of Src-

induced activation of the PI3K-Akt signal transduction pathway during TRAIL treatment. Cell Signal 2010; 22(3):377-385.
92. Song JJ, An JY, Kwon YT, Lee YJ. Evidence for two modes of development of acquired tumor necrosis factor-related apoptosis-inducing ligand resistance. Involvement of Bcl-xL. J Biol Chem 2007; 282(1):319-328.
93. Lalaoui N, Morle A, Merino D, Jacquemin G, Iessi E, Morizot A et al. TRAIL-R4 promotes tumor growth and resistance to apoptosis in cervical carcinoma HeLa cells through AKT. PLoS One 2011; 6(5):e19679.
94. De Toni EN, Kuntzen C, Gerbes AL, Thasler WE, Sonuc N, Mucha SR et al. P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. J Hepatol 2007; 46(4):682-691.
95. Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M et al. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell 2009; 16(1):67-78.
96. Cursi S, Rufini A, Stagni V, Condo I, Matafora V, Bachi A et al. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. EMBO J 2006; 25(9):1895-1905.
97. Van Schaeybroeck S, Kelly DM, Kyula J, Stokesberry S, Fennell DA, Johnston PG et al. Src and ADAM-17-mediated shedding of transforming growth factor-alpha is a mechanism of acute resistance to TRAIL. Cancer Res 2008; 68(20):8312-8321.
98. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol 2002; 168(3):1356-1361.
99. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI et al. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J Clin Invest 2008; 118(1):100-110.
100. Ishimura N, Isomoto H, Bronk SF, Gores GJ. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 2006; 290(1):G129-G136.
101. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006; 25(56):7434-7439.
102. Kim JH, Choi C, Benveniste EN, Kwon D. TRAIL induces MMP-9 expression via ERK activation in human astrocytoma cells. Biochem Biophys Res Commun 2008; 377(1):195-199.
103. Li Z, Xu X, Bai L, Chen W, Lin Y. Epidermal growth factor receptor-mediated tissue transglutaminase overexpression couples acquired tumor necrosis factor-related apoptosis-inducing ligand resistance and migration through c-FLIP and MMP-9 proteins in lung cancer cells. J Biol Chem 2011; 286(24):21164-21172.
104. Zhou DH, Trauzold A, Roder C, Pan G, Zheng C, Kalthoff H. The potential molecular mechanism of overexpression of uPA, IL-8, MMP-7 and MMP-9 induced by TRAIL in pancreatic cancer cell. Hepatobiliary Pancreat Dis Int 2008; 7(2):201-209.
105. Ehrenschwender M, Siegmund D, Wicovsky A, Kracht M, ttrich-Breiholz O, Spindler V et al. Mutant PIK3CA licenses TRAIL and CD95L to induce non-apoptotic caspase-8-mediated ROCK activation. Cell Death Differ 2010; 17(9):1435-1447.
106. Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, Westendorp BF et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 2010; 138(7):2357-2367.
107. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
108. Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D et al. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt

and ERK pathways. Circulation 2003; 107(17):2250-2256.
109. Li JH, Kirkiles-Smith NC, McNiff JM, Pober JS. TRAIL induces apoptosis and inflammatory gene expression in human endothelial cells. J Immunol 2003; 171(3):1526-1533.
110. Secchiero P, Corallini F, di Iasio MG, Gonelli A, Barbarotto E, Zauli G. TRAIL counteracts the proadhesive activity of inflammatory cytokines in endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release. Blood 2005; 105(9):3413-3419.
111. Wilson NS, Yang A, Yang B, Couto S, Stern H, Gogineni A et al. Proapoptotic activation of death receptor 5 on tumor endothelial cells disrupts the vasculature and reduces tumor growth. Cancer Cell 2012; 22(1):80-90.
112. Secchiero P, Zerbinati C, Rimondi E, Corallini F, Milani D, Grill V et al. TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells. Cell Mol Life Sci 2004; 61(15):1965-1974.
113. Kavurma MM, Schoppet M, Bobryshev YV, Khachigian LM, Bennett MR. TRAIL stimulates proliferation of vascular smooth muscle cells via activation of NF-kappaB and induction of insulin-like growth factor-1 receptor. J Biol Chem 2008; 283(12):7754-7762.
114. Chan J, Prado-Lourenco L, Khachigian LM, Bennett MR, Di Bartolo BA, Kavurma MM. TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. Circ Res 2010; 106(6):1061-1071.
115. Chou AH, Tsai HF, Lin LL, Hsieh SL, Hsu PI, Hsu PN. Enhanced proliferation and increased IFN-gamma production in T cells by signal transduced through TNF-related apoptosis-inducing ligand. J Immunol 2001; 167(3):1347-1352.
116. Robertson NM, Zangrilli JG, Steplewski A, Hastie A, Lindemeyer RG, Planeta MA et al. Differential expression of TRAIL and TRAIL receptors in allergic asthmatics following segmental antigen challenge: evidence for a role of TRAIL in eosinophil survival. J Immunol 2002; 169(10):5986-5996.
117. Morel J, Audo R, Hahne M, Combe B. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rheumatoid arthritis synovial fibroblast proliferation through mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt. J Biol Chem 2005; 280(16):15709-15718.
118. Rimondi E, Secchiero P, Quaroni A, Zerbinati C, Capitani S, Zauli G. Involvement of TRAIL/TRAIL-receptors in human intestinal cell differentiation. J Cell Physiol 2006; 206(3):647-654.
119. Wu NL, Lee TA, Tsai TL, Lin WW. TRAIL-induced keratinocyte differentiation requires caspase activation and p63 expression. J Invest Dermatol 2011; 131(4):874-883.
120. Yen ML, Tsai HF, Wu YY, Hwa HL, Lee BH, Hsu PN. TNF-related apoptosis-inducing ligand (TRAIL) induces osteoclast differentiation from monocyte/macrophage lineage precursor cells. Mol Immunol 2008; 45(8):2205-2213.
121. Kim JY, Lee JY, Kim DG, Koo GB, Yu JW, Kim YS. TRADD is critical for resistance to TRAIL-induced cell death through NF-kappaB activation. FEBS Lett 2011; 585(14):2144-2150.
122. Lluis JM, Nachbur U, Cook WD, Gentle IE, Moujalled D, Moulin M et al. TAK1 is required for survival of mouse fibroblasts treated with TRAIL, and does so by NF-kappaB dependent induction of cFLIPL. PLoS One 2010; 5(1):e8620.
123. Cao X, Pobezinskaya YL, Morgan MJ, Liu ZG. The role of TRADD in TRAIL-induced apoptosis and signaling. FASEB J 2011; 25(4):1353-1358.
124. Jurewicz A, Matysiak M, Andrzejak S, Selmaj K. TRAIL-induced death of human adult oligodendrocytes is mediated by JNK pathway. Glia 2006; 53(2):158-166.

Chapter 3

MAPK p38 and JNK have opposing activities on TRAIL-induced apoptosis activation in NSCLC cells that involves RIP1 and caspase-8 and is mediated by Mcl-1

Kaamar Azijli, Saravanan Yuvaraj, Ingrid van Roosmalen, Koen Flach, Elisa Giovannetti, Godefridus J. Peters, Steven de Jong, Frank A.E. Kruyt

Apoptosis (Accepted, in press)

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce both caspase-dependent apoptosis and kinase activation in tumor cells. Here, we examined the consequences and mechanisms of TRAIL-induced MAPKs p38 and JNK in non-small cell lung cancer (NSCLC) cells. In apoptosis sensitive H460 cells, these kinases were phosphorylated, but not in resistant A549 cells. Time course experiments in H460 cells showed that induction of p38 phosphorylation preceded that of JNK. To explore the function of these kinases in apoptosis activation by TRAIL, chemical inhibitors or siRNAs were employed to impair JNK or p38 functioning. JNK activation counteracted TRAIL-induced apoptosis whereas activation of p38 stimulated apoptosis. Notably, the serine/threonine kinase RIP1 was cleaved following TRAIL treatment, concomitant with detectable JNK phosphorylation. Further examination of the role of RIP1 by short hairpin (sh)RNA-dependent knockdown or inhibition by necrostatin-1 showed that p38 can be phosphorylated in both RIP1-dependent and -independent manner, whereas JNK phosphorylation occurred independent of RIP1. On the other hand JNK appeared to suppress RIP1 cleavage via an unknown mechanism. In addition, only the activation of JNK by TRAIL was caspase-8-dependent. Finally, we identified Mcl-1, a known substrate for p38 and JNK, as a downstream modulator of JNK or p38 activity. Collectively, our data suggest in a subset of NSCLC cells a model in which TRAIL-induced activation of p38 and JNK have counteracting effects on Mcl-1 expression leading to pro- or anti-apoptotic effects, respectively. Strategies aiming to stimulate p38 and inhibit JNK may have benefit for TRAIL-based therapies in NSCLC.

Key Words: TRAIL, kinases, combination therapy, lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) accounting for about 80% of all lung cancer cases [1]. Most patients are diagnosed at an advanced metastasized stage of disease, precluding local surgery in most cases. The standard treatment regime for advanced stage NSCLC includes both platinum-based chemotherapy and thoracic radiotherapy [2]. However, the median survival on patients treated with these standard regimens ranges from 11 to 18 months [3]. Furthermore, these treatments are associated with high toxicity. Novel and less toxic treatment strategies to improve survival of these patients are therefore urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which belongs to the TNF super-family, is a promising candidate for the treatment of cancer, including NSCLC. TRAIL induces apoptosis in cancer cells, while leaving normal cells intact. It can bind to five different receptors, also named death receptors (DR), of which TRAIL-R1 (DR4) and TRAIL-R2 (DR5) mediate the induction of apoptosis. TRAIL can also bind to decoy receptors TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and the soluble receptor osteoprotegerin (OPG) that compete for TRAIL ligation and are thought to suppress apoptosis activation [4]. Following binding of TRAIL to TRAIL-R1 or –R2 several proteins are recruited to death domains present in the intracellular portion of the receptors facilitating the formation of the death-inducing signaling complex (DISC). In the DISC, FADD and caspase-8 are recruited and activated caspase-8 can subsequently activate downstream effector caspases triggering the execution of apoptosis. In type I cells, levels of caspase-8 activation are sufficient to directly activate the effector caspases, such as caspase-3. In type II cells, stimulation of the mitochondrial apoptotic pathway is essential for full effector caspases activation. This is achieved via caspase-8-dependent cleavage of Bid, producing truncated (t)Bid that results in activation of the pro-apoptotic Bcl-2 family members, BAX or BAK. These proteins cause induction of mitochondrial outer membrane permeabilization (MOMP) leading to cytosolic release of apoptogenic factors such as cytochrome c, which is a cofactor for caspase-9 activation leading to effector caspases activation [5].

In addition to the DISC, also a secondary complex can be formed following TRAIL receptor activation that has been associated with non-apoptotic signaling [6]. This secondary complex, consisting of RIP1 kinase, TRAF2, NEMO/IKKγ, FADD, and caspase-8, can activate several downstream signaling pathways that are involved in cell survival and cell proliferation, including activation of MAPK-dependent signaling [5]. Even more, in some resistant tumor cells TRAIL can enhance their migratory and invasive activity [7;8], as was recently demonstrated in resistant NSCLC cells in a RIP1-dependent manner [9]. Inhibition of TRAIL-induced kinase activation may thus be required in order to enhance the apoptotic properties of TRAIL, while counteracting possible unwanted protumorigenic effects of TRAIL. A number of studies have shown that inhibition of MAPK pathways can sensitize some TRAIL-resistant tumor cells for apoptosis suggesting that the combined use of MAPK

inhibitors and TRAIL receptor targeting agents may be an opportunity for cancer therapy [10-16]. In the present study, we have examined TRAIL-induced activation of the MAPKs p38 and JNK in TRAIL sensitive and resistant NSCLC cells. The consequences and mechanism of activation have been examined indicating opposing effects of these two kinases on TRAIL-induced apoptosis.

MATERIALS & METHODS

Cell Culture

NSCLC cells A549, H460, H1299, H1975, H322, and SW1573 were cultured as monolayers in RPMI 1640 medium supplemented with (v/v) 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Cell death measurement

Cell death measurements were performed by FACS analysis as described previously [9]. Cells were seeded in 6-well plates at a density of 300,000 cells/well. After drug exposure, the adherent NSCLC cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1200 rpm. Next, cells were stained with propidium iodide (PI) buffer (50 µg/ml PI, 0.1 mg/ml RNAse A, 0.1% Triton-X, 0.1% (Tri-) Sodium Citrate dissolved in PBS) in the dark on ice. DNA content of the cells was analyzed by a FACSCalibur flowcytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G_1 peak was used to determine the extent of cell death.

Western blotting

Western blotting was performed as described previously [9]. Briefly, cells were exposed to TRAIL for different time points or pre-incubated with p38 and JNK inhibitors for 30 min prior to TRAIL treatment. The cells were washed twice with ice-cold PBS and resuspended in lysis buffer (Cell Signalling Technology Inc.) supplemented with 0.04% protease inhibitor cocktail (Roche, Almere, the Netherlands) and Na_2VO_3. Cell lysates were scraped, transferred into a vial and centrifuged at 11,000 g at 4 °C for 10 min. Protein concentrations were determined by the Bio-Rad assay, according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the Netherlands). The following primary antibodies were used all from Cell Signaling Technology Inc. (Danvers, MA, USA) at a 1:1000 dilution: Anti-caspase 3 (#9662), anti-caspase 8 (#9746), anti-caspase 9 (#9502), anti-cleaved caspase 3 (#9661), phospho-SAPK/JNK (Thr183/Tyr185, #9251), SAPK/JNK (#9252), phospho-p38 (Thr180/Tyr182, #9211), p38 (#9212), Mcl-1 (#4572), RIP1 (#3493), PARP (#9542), Bid (#2002), Bcl-2 (#2872), Bcl-xL (#2762). As secondary antibodies (1:10,000 goat-α-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) or goat-α-

rabbit-IRDye (800CW;926-32211 and 680;#926-32221) were used. Protein expression was visualized with the Odyssey Infrared Imager (Westburg, Leusden, the Netherlands), at a 84 μm resolution, 0 mm offset and with high quality [17].

RNA interference

H460 cells were plated at a density of 7.5 x10^4 cells in 6-well tissue culture plates and allowed to attach overnight. The next day, cells were incubated with serum-free medium and transfected with 100 nM SignalSilence® p38 MAPK siRNA I (#6564), 100 nM SignalSilence® SAPK/JNK siRNA I (#6232), SignalSilence® Control siRNA (Fluorescein Conjugate) (#6201) (all from Cell Signaling Technology Inc), 20 pmol Mcl-1 siRNA (Dharmacon, M-004501-08) or scrambled siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were then incubated for a minimum of 24 h before assaying for expression.

For short hairpin (sh)RNA mediated silencing pSUPER.retro plasmid was used similarly as described previously [18]. Targeted shRNA sequences were inserted into the BglII and HindIII sites of the pSUPER.retro vector. All cloned shRNA sequences were verified by DNA sequencing. Retroviruses were packaged and introduced into cells as described previously [19]. H460 cells were retrovirally infected with control pSUPER.retro or pSUPER.retro-shRIP1 (RIP1-targeting sequence #1, 5'- GAGCAGCAGTTGATAATGT-3'; RIP1-targeting sequence #2 5'- TACCACTAGTCTGACGGATAA-3') for 24 h. Infected cells were selected with 2 μg/ml puromycin.

Quantitative RT-PCR

Total RNA was extracted using the QiaAmp RNA mini-Kit (Qiagen, San Diego, CA), and yields and purity were checked at 260-280 nm with NanoDrop®-1000-Detector (NanoDrop-Technologies, Wilmington, NC). RNA (500 ng) was reverse transcribed using the DyNAmo cDNA Synthesis Kit (Thermo Scientific, Vantaa, Finland), according to the manufacturers' instruction. Primers and probes to specifically amplify Mcl-1 and Bcl-2 were obtained from Applied Biosystems Assay-on-Demand Gene expression products (Hs01050896_m1, and Hs00608023_m1). The real-time quantitative PCR was performed in a 25 μl reaction volume containing TaqMan Universal master mix (Applied Biosystems, Forster City, CA). All reactions were performed in triplicate using the ABI PRISM 7500 sequence detection system instrument (Applied Biosystems). Samples were amplified using the following thermal profile: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 sec followed by annealing and extension at 60 °C for 1 min. Amplifications were normalized to β-actin, whose values were the closest to the geometric mean values observed for three housekeeping genes in preliminary analysis. Preliminary experiments were carried out with dilutions of cDNA obtained from Quantitative PCR Human Reference Total RNA (Stratagene, La Jolla, CA) to demonstrate that the efficiencies

of amplification of the target and reference genes are approximately equal and to determine the absolute value of the slope of standard cDNA concentration versus CT, where CT is the threshold cycle, as reported previously [20].

RESULTS

TRAIL-dependent activation of p38 and JNK MAPKs in NSCLC cells

TRAIL-induced p38 and JNK phosphorylation were studied in H460 and A549 cells representing TRAIL sensitive and resistant cells, respectively [21]. Time course experiments applying TRAIL (50 ng/ml) for up to 4 h confirmed strong caspase-8, -9 and -3 cleavage in H460 cells already after 1 h exposure. A549 cells showed only limited caspase cleavage, which was not sufficient for triggering apoptosis (Fig. 1a). These results were in line with hardly any increase in caspase activity after TRAIL treatment in A549 cells contrasting strong activation in H460 cells (data not shown). Using the same cell lysates TRAIL-induced phosphorylation of p38 and JNK were examined. In H460 cells, p38 and JNK were phosphorylated after 2 h and 3 h TRAIL exposure, respectively, whereas no phosphorylation was seen in A549 cells (Fig. 1b). Since RIP1 kinase has been reported to mediate MAPK activation [22], we determined RIP1 expression in the NSCLC cells. Both A549 and H460 expressed RIP1, but only H460 cells displayed clear TRAIL-induced RIP1 cleavage that was detectable at around 30 min post-treatment and with strong cleavage after 2 h treatment.

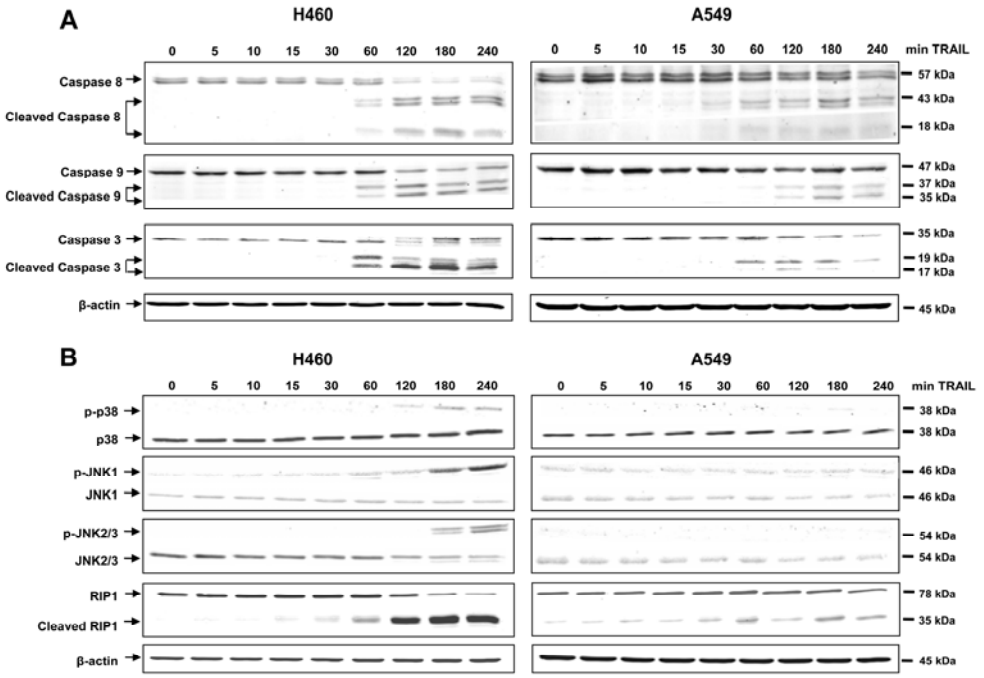

Figure 1. Caspases activation, JNK and p38 phosphorylation and RIP expression in TRAIL-sensitive H460 and -resistant A549 cells. (**a**) The H460 and A549 cell lines were treated with 50 ng/ml TRAIL for the indicated time points and caspase-8, -9, -3 cleavage was assessed by Western blotting. (**b**) Phosphorylation of p38 (p-p38) and phosphorylation of JNK1/2/3 (p-JNK1/2/3), as well as RIP1 expression following TRAIL treatment (50 ng/ml) were determined by Western blotting. The blots are representative for at least three independent experiments.

P38 and JNK have opposing effects on TRAIL-induced apoptosis

P38 and JNK have been described to mediate both pro- and anti-apoptotic activities depending on the stimulus applied and the cell type studied [23]. In order to explore their role in TRAIL-induced apoptosis in NSCLC cells we employed selective chemical JNK and p38 inhibitors. As shown in Fig. 2a, JNK inhibition with SP600125 resulted in enhanced TRAIL-induced cell death in H460 cells, indicating anti-apoptotic activity of JNK. P38 inhibition with SB203580, on the other hand, decreased the level of cell death, indicating that p38 has pro-apoptotic activity in TRAIL signaling (Fig. 2b). The chemical inhibitors SP600125 and SB203580 have been generated against the ATP binding pockets of JNK and p38, respectively, and do not affect the phosphorylation status of the kinases themselves or only partially in case of autophosphorylation [24;25]. As a control and in line with this we did not observe a decrease in TRAIL-dependent phosphorylation of JNK and p38 in the presence of these inhibitors (Fig. 2f). Furthermore, at concentrations lower than 25 μM hardly any significant cross-inhibition of other MAPKs have been reported, which has been linked to the unique binding properties of these chemicals in the ATP-binding sites of the enzymes [26]. Nonetheless, to further corroborate our findings we also employed specific siRNAs against JNK and p38 that upon transfection strongly reduced the expression of these kinases in H460 cells when compared to control siRNA transfected cells (Fig 2c, d). In resistant A549 cells, JNK and p38 inhibition did not significantly affect TRAIL-dependent apoptosis (Fig. 2a, b). Next, the effect of kinases inhibition on TRAIL-induced caspases activation was studied by Western blotting following 3 or 24 h treatment. JNK inhibition by SP600125 appeared to stimulate caspase-8, Bid, RIP1, caspase-9, caspase-3 and PARP cleavage, as judged by more intense cleaved products and/ or a decrease in the full length proteins (Fig. 2e). Inhibition of p38 with SB203580 in combination with TRAIL hardly affected cleavage of caspase-8, Bid and RIP1, whereas cleavage of caspase-9, caspase-3 and PARP was somewhat decreased compared to TRAIL alone.

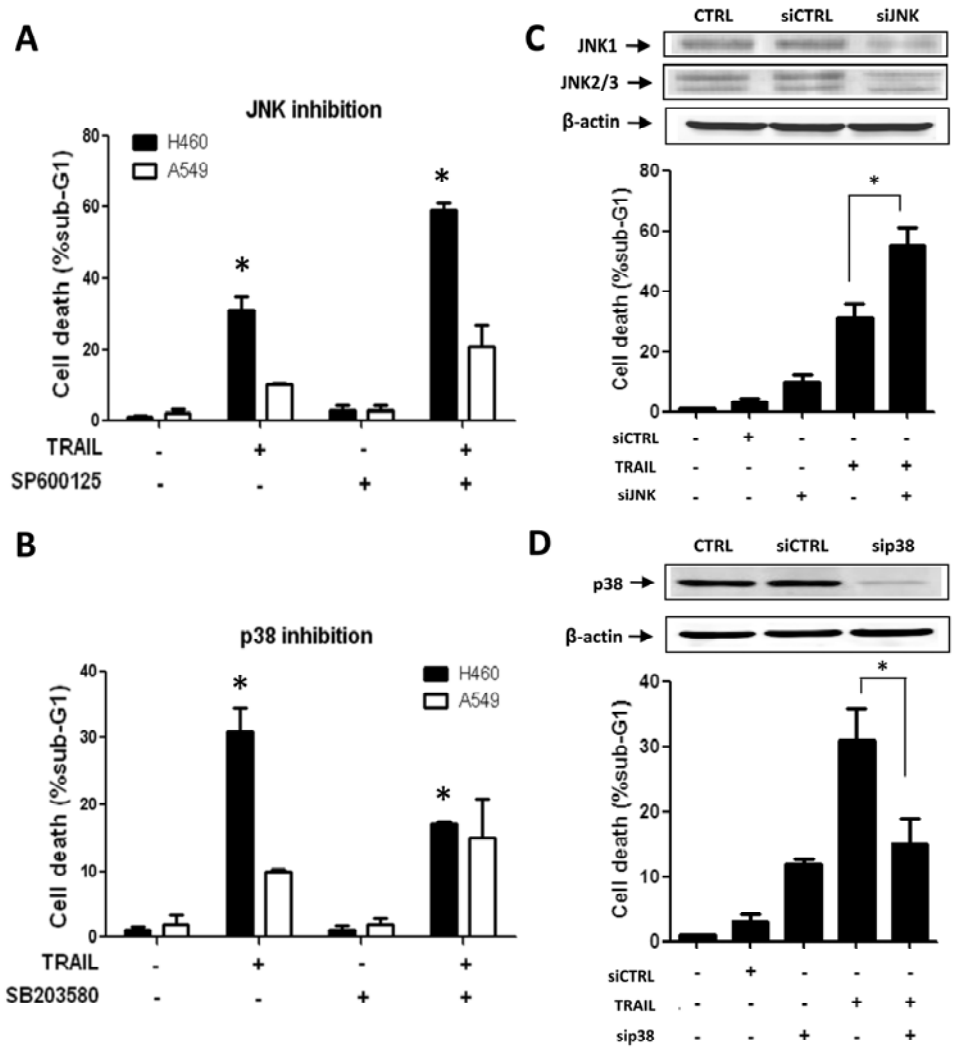

Figure 2. JNK suppresses - and p38 stimulates TRAIL-induced apoptosis in H460 cells. (**a, b**) H460 cells were treated with 50 ng/ml TRAIL for 24 h with or without the JNK inhibitor, SP600125 (10 μM) or the p38 inhibitor, SB203580 (10 μM). Cell death was analyzed by determining the sub-G1 fraction by FACS of PI-stained cells. The averages of experimental triplicates (± s.d.) are shown in **a** and **b**. *p<0.05, t-test compared to each other. (**c**) The expression of JNK (**d**) or p38 in H460 cells was silenced with specific siRNA for 72h and compared to control siRNA. In addition, cell death (%sub-G1) in H460 cells controls and after JNK or p38 silencing for 72h was determined following exposure to TRAIL (50ng/ml) for the last 24 h. The experiments were performed twice, *p<0.05, t-test.

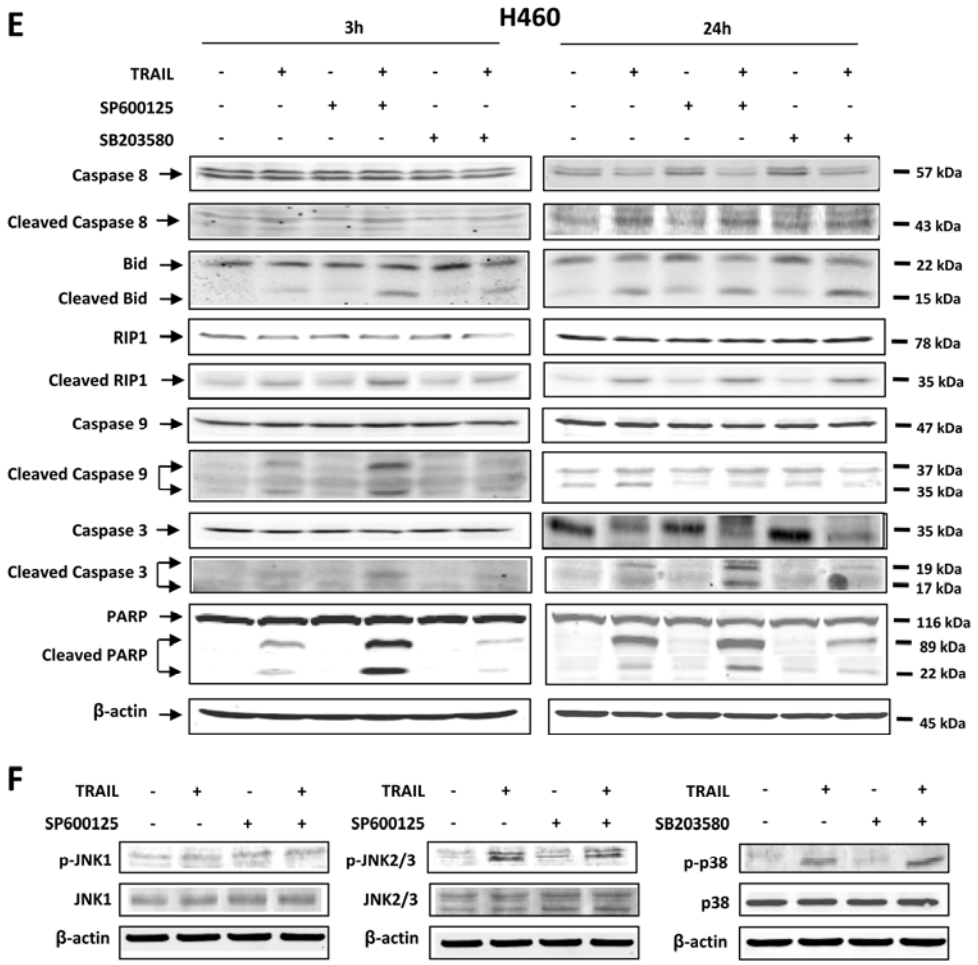

Figure 2 (continued). (**e**) Expression and cleavage of caspase-8, Bid, RIP1, caspase-9, caspase-3, PARP in H460 cells after 3 h and 24 h incubation with 50 ng/ml TRAIL. (**f**) H460 cells were treated with SP600125 (10 μM) or SB203580 (10 μM) with or without TRAIL for 3h and the phosphorylation status of JNK and p38 were determined.

Effect of RIP1 on TRAIL-induced caspases activation and p38 and JNK phosphorylation

Next, we further explored the role of RIP1 in mediating p38 and JNK signaling in NSCLC cells. In H460 cells, RIP1 expression was reduced using a specific shRNA leading to an increased amount of cell death after 24 h TRAIL treatment when compared to vector control cells, revealing its contribution to prosurvival effects (Fig. 3a). JNK inhibition by SP600125 further increased the amount of cell death in these cells. The decrease in TRAIL-induced cell death in H460 cells by SB203580-mediated p38 inhibition was not observed in H460-shRIP1 cells. In agreement with stronger apoptosis activation in RIP1-silenced cells an increase in caspase-8, -9, and -3 cleavages were detected (Fig. 3b). Unexpectedly,

enhanced phosphorylation of both p38 and JNK were seen upon RIP1 knockdown (Fig. 3c), suggesting that RIP1 to some extent can suppress the activation of these kinases by TRAIL. Similarly, treatment with the selective RIP1 kinase inhibitor necrostatin-1 [27] also enhanced TRAIL-induced phosphorylation of JNK and p38 (Fig. 3d). However, as found, activation of p38 under RIP1 knockdown conditions does not contribute to apoptosis activation, contrasting its pro-apoptotic activity in the presence of RIP1.

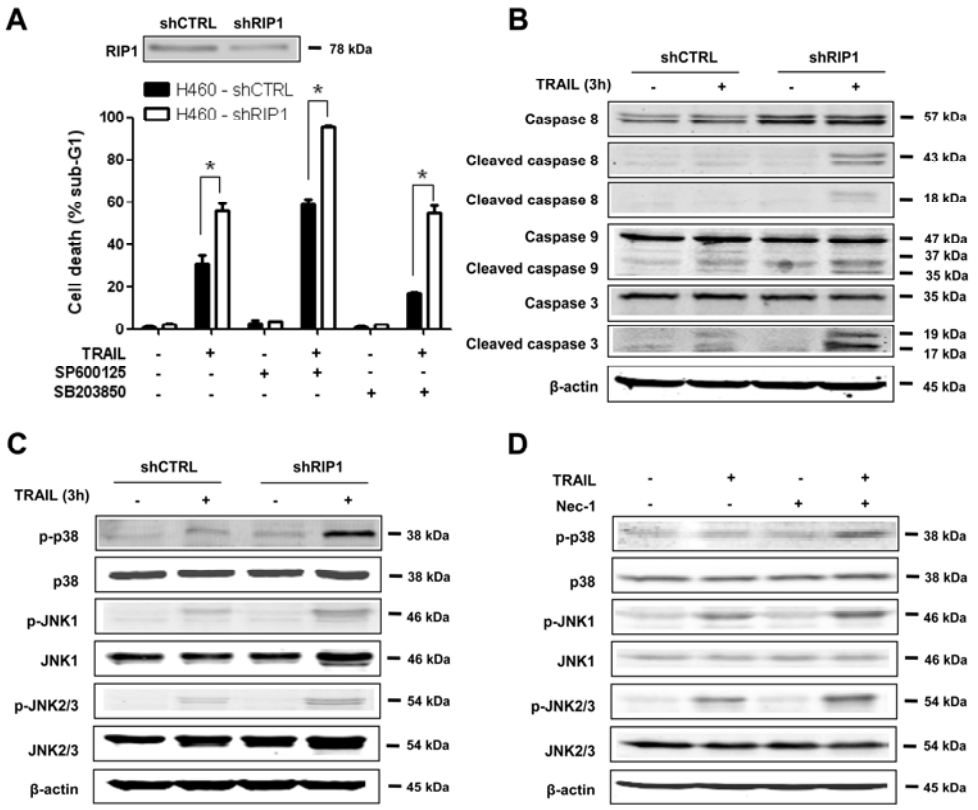

Figure 3. RIP1 knockdown enhances TRAIL-induced apoptosis and affects p38 and JNK phosphorylation in H460 cells. (**a**) H460-shCTRL and H460-shRIP1 cells were treated with TRAIL (50 ng/ ml) in absence or presence of 10 μM SP600125 or 10 μM SB203580. *p<0.05, t-test. Inset, immunoblot showing RIP1 reduction in H460-shRIP1 cells compared to control. (**b**) TRAIL-induced caspase cleavage and (**c**) p38 and JNK phosphorylation in H460-shCTRL and H460-shRIP1 cells and (**d**) in H460 cells after incubation with the RIP1 inhibitor Necrostatin-1 (Nec-1, 30 μM). The cells were preincubated for 1 h with Nec-1 before stimulation with TRAIL for 3h.

Caspase-8 dependency of JNK and p38 phosphorylation

Previously, Varfolomeev and co-workers have shown that the presence of caspase-8 in the secondary complex is required for effective kinase activation by TRAIL, including p38 and JNK [22]. In order to study the role of caspase-8 in the activation of p38 and JNK we used a

previously established H460-derived cell line, H460-CrmA [28]. In this cell line, the cowpox virus-encoded anti-apoptotic protein cytokine response modifier A (CrmA), an inhibitor of caspase-8, is stably overexpressed. Apoptosis by TRAIL was efficiently prevented in CrmA expressing cells and inhibition of p38 or JNK under these conditions had no effect, as depicted in Fig. 4a. As expected, TRAIL-induced caspase-8 and RIP1 cleavage was not observed in the H460-CrmA cells as shown in Fig. 4b. Furthermore, inhibition of caspase-8 activation did not affect p38 phosphorylation after TRAIL exposure (Fig. 4c). However, TRAIL-induced JNK phosphorylation was not detected in H460-CrmA cells, indicating that caspase-8 activity is important for JNK activation by TRAIL.

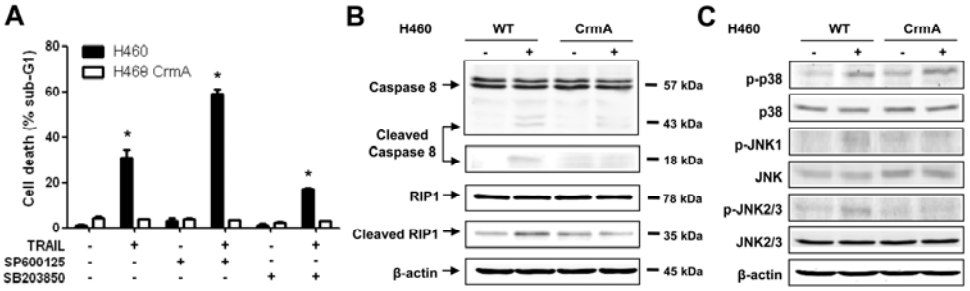

Figure 4. TRAIL-induced caspase-8 activation is required for JNK phosphorylation. (**a**) H460 cells overexpressing the caspase-8 inhibiter CrmA (H460-CrmA) were treated with and without TRAIL alone, or in combination with SP600125 (10 μM) or SB203580 (10 μM) for 24 h. Cell death was determined by FACS analysis of the sub-G1 fraction. Means of triplicate experiments (± s.d.) are shown. (**b**) Caspase-8 and RIP1 cleavage by TRAIL in H460 and H460-CrmA cells. (**c**) Western blots displaying p38 and JNK and phosphorylated variants. The cells were treated with 50 ng/ml TRAIL for 3 h.

Mcl-1 is a mediator of pro-apoptotic and anti-apoptotic effects of p38 and JNK, respectively.

Anti-apoptotic Mcl-1 and Bcl-2 are known transcriptional targets of both p38 and JNK signaling [23;29;30]. Therefore, we studied the effect of p38 and JNK inhibition on Mcl-1 and Bcl-2 mRNA levels using qPCR in H460 cells. As shown in Fig. 5a, SP600125 treatment decreased Mcl-1 expression, whereas p38 inhibition by SB203580 enhanced transcription. Bcl-2 transcription was not affected by these treatments. Examination of protein levels by Western blotting revealed a reduction in Mcl-1 expression upon JNK inhibition, while p38 inhibition clearly enhanced Mcl-1 expression (Fig. 5b). Similar results were obtained when silencing JNK and p38 expressions with specific siRNAs (not shown). It thus appears that normally JNK activity enhances Mcl-1 expression, while in contrast p38 activity reduces Mcl-1 levels. To further explore the role of Mcl-1 in this context its expression was silenced in H460 cells using a specific siRNA (Fig. 5c). In these Mcl-1 knockdown cells TRAIL-induced cell death was enhanced by approximately 100% when compared to control H460 cells.

Notably, Mcl-1 knockdown cells were not responding to either the pro-apoptotic effect of JNK inhibition or anti-apoptotic effect of p38 inhibition, respectively (Fig. 5d). This indicates that Mcl-1 is a mediator of the apoptosis modulatory effects of p38 and JNK.

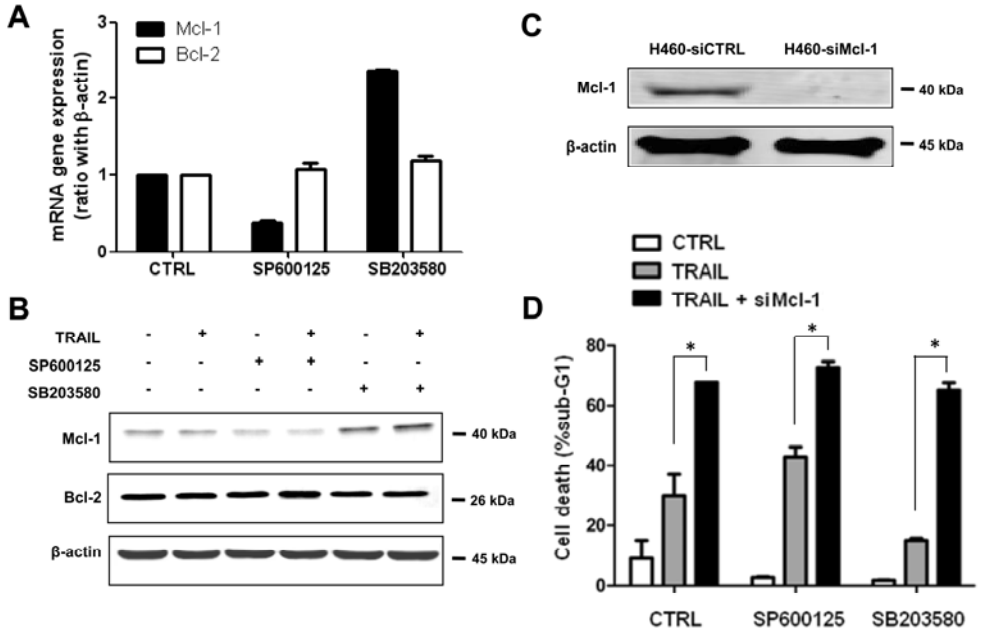

Figure 5. Mcl-1 is a mediator of the anti-apoptotic effect of JNK and pro-apoptotic effect of p38 in TRAIL signaling. (**a**) Quantitative RT-PCR was performed to determine mRNA expression of Mcl-1 and Bcl-2 after exposure to 10 μM SP600125 or 10 μM SB203580 for 24 h in H460 cells. (**b**) Mcl-1 and Bcl-2 expression in H460 cells treated with TRAIL (50 ng/ml) ± SP600125 (10 μM) or ± SB203580 (10 μM) for 24 h. (**c**) Mcl-1 expression was silenced using siRNA in H460 cells and the expression levels were determined by Western blotting and compared with control shRNA transfected cells. (**d**) H460-shCTRL and H460-shMcl-1 cells were treated with TRAIL (50 ng/ml) with and without SP600125 (10 μM) or SB203580 (10 μM) and effect on apoptosis was determined.

P38 and JNK activation in a panel of NSCLC cells

Finally, the effect of p38 and JNK inhibition on TRAIL sensitivity was examined in an extended panel of NSCLC cells. TRAIL sensitivity was variable in these cells (Fig. 6a). H1299 and SW1573 cells showed less than 5% apoptosis when corrected for background apoptosis levels. H322 cells displayed intermediate sensitivity with approximately 10% apoptosis, whereas H1975 cells with around 25% apoptosis were most sensitive. Co-incubation with the JNK inhibitor SP600125 led to an increase in the amount of cell death, mainly in the H1975 and H322 cell lines, in which the increase in sub-G1 levels was around 8% and 6%, respectively. The increase in TRAIL-induced cell death by SP600125 was only 2% in H322 cells and 3% in SW1573 cells. The p38 inhibitor SB203850 did have little or no

effect on TRAIL-induced cell death in these cells.

TRAIL-induced phosphorylation of p38 and JNK was also studied in these cell lines. Cells were treated with 50 ng/ml TRAIL for 3 h and expressions of p38 and JNK and their phosphorylated forms were evaluated by Western blotting (Fig. 6b). Pleiotropic responses were observed, with clear p38 phosphorylation only in resistant H1299 cells. In these cells inhibition of p38 did not affect apoptosis (Fig. 6a) indicating that in the absence of TRAIL-dependent apoptotic signaling modulation of p38 activity alone has no apparent effect on apoptosis. JNK phosphorylation was detected in H1975, H322, and SW1573 cells following TRAIL treatment. In these three cell lines RIP1 cleavage by TRAIL could be seen. Taken together, inhibition of JNK has a TRAIL apoptosis stimulatory effect in NSCLC displaying already sensitivity to TRAIL-dependent apoptosis.

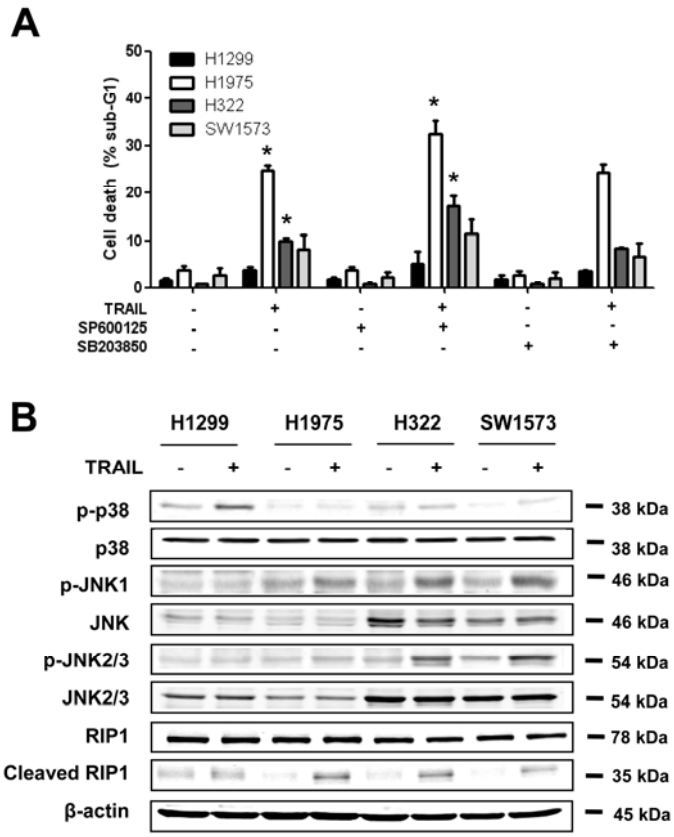

Figure 6. Effect of p38 and JNK inhibition on TRAIL-induced apoptosis in a panel of NSCLC cells. (**a**) H1299, H1975, H322, and SW1573 cells were exposed to TRAIL (50 ng/ml) ± SP600125 (10 μM) or ± SB203580 (10 μM) for 24h. Cell death was determined as percentage of sub-G1 cells. *p<0.05, t-test, compared to each other (**b**) Lysates generated from treated cells (50 ng/ml TRAIL for 3 h) were examined for expression of p-p38, p38, p-JNK1/2/3, JNK1/2/3 and RIP1 by Western blotting. The experiments were performed at least twice.

DISCUSSION

Tumor cells, including NSCLC cells, display variable sensitivity to TRAIL receptor-targeted agents ranging from highly sensitive, intermediate sensitive to highly resistant [31;32]. In this study, we aimed to examine the role of MAPK p38 and JNK in modulating the apoptotic response in NSCLC cells and to elucidate the underlying mechanisms. JNK and p38 were phosphorylated within a few hours in sensitive H460 cells, but not in resistant A549 cells (Fig. 1). In H460 cells, co-exposure of TRAIL with chemical inhibitors of JNK and p38 or siRNAs against these kinases identified a proapoptotic function of p38, contrasting a prosurvival effect of JNK (Fig. 2). In a broader NSCLC cell panel with varying TRAIL sensitivity, JNK inhibition enhanced apoptosis particularly in the TRAIL intermediate sensitive H1975 and H322 cells, confirming antiapoptotic activity of this kinase (Fig. 6). Inhibition of p38 did not significantly affect TRAIL sensitivity.

The mechanism by which JNK and p38 affect apoptosis by TRAIL in H460 cells was studied in more detail. We found that TRAIL-induced p38 phosphorylation preceded JNK phosphorylation (Fig. 1). Interestingly, JNK activation occurred when RIP1 cleavage was evident. Furthermore, comparison of caspase cleavage patterns in TRAIL and TRAIL/ p38 inhibitor-treated H460 cells indicated a stimulatory effect of p38 on caspase-9 and -3 and PARP cleavage (Fig. 2e). Similar experiments with the JNK inhibitor revealed a suppressive effect of JNK on the cleavage of caspase-8, Bid, caspase-9, -3 and PARP, and also RIP1 cleavage. These findings suggest a role of both p38 and JNK in affecting mitochondrial/ caspase-9-dependent apoptosis, whereas JNK inhibition has additive effects on the activation of the DISC compounds RIP1 and caspase-8, and subsequent engagement of the mitochondrial route via Bid cleavage. Part of the prosurvival effect of JNK may be the suppression of RIP1 cleavage, and RIP1 on its turn being able to prevent TRAIL-dependent JNK activation in some sort of feedback loop. However, thus far RIP1 is not a known substrate of JNK [33] and the effect of JNK on RIP1 cleavage may be indirect requiring another protein.

RIP kinases constitute a family of seven members, namely RIP1-7. They are serine threonine kinases being crucial regulators of cell survival and cell death. Depending on the cellular context, RIP1 is implicated in the activation of NF-κB, MAPKs, apoptosis or necrosis [34]. RIP1 can be cleaved by caspase-8, abrogating NF-κB activation and together with the cleaved product, RIPc, interacts with TRADD and FADD, leading to an increase of death receptor-induced apoptosis [35;36]. We found that reduction of RIP1 expression by a specific shRNA or inhibition of its kinase activity with necrostatin-1 enhanced TRAIL-induced apoptosis in H460 cells that could be augmented by JNK inhibition. This indicates that the anti-apoptotic activity of JNK was maintained in the absence of RIP1 activity (Fig. 3). Indeed, TRAIL-induced phosphorylation of JNK remained detectable in RIP1 knockdown cells. Interestingly, phosphorylation of p38 was enhanced but did not have pro-apoptotic activity under these conditions, since p38 inhibition did not alter the level of TRAIL-

induced apoptosis in H460-shRIP1 cells. The molecular mechanism underlying this finding is yet unclear, but may point to a role for RIP1 in determining the substrate specificity of p38 and thus the functional consequences.

TRAIL-induced JNK phosphorylation required caspase-8 cleavage and activation as it was suppressed in H460-CrmA cells, whereas TRAIL binding is sufficient to induce p38 phosphorylation (Fig. 4). Together, these findings reflect our observation that JNK phosphorylation occurs at the time that RIP1 is cleaved and inactivated. P38 phosphorylation remains present also after RIP1 is cleaved, in accordance with the finding that p38 can be activated both in the presence and absence of RIP1 (Fig. 3c). In an earlier study it was reported that in HeLa cells and mouse fibroblasts RIP1 and TRAF2 are required for TRAIL-mediated JNK activation [37]. However, another study showed that in fibrosarcoma cells RIP1 is not essential for TRAIL-induced JNK stimulation and that on the other hand p38 activation was RIP1-dependent [22]. Thus, the role of RIP1 in TRAIL-induced JNK and p38 activation appears to vary in a cell-specific manner.

We identified Mcl-1 as a major mediator of both JNK and p38 activity leading to enhanced or decreased Mcl-1 expression, respectively. A role for Mcl-1 as an apoptotic modulator of TRAIL-induced p38 and JNK activation has been described earlier in TRAIL resistant prostate cancer cells [29]. However, contrary to our findings in NSCLC, activation of both kinases led to transcriptional upregulation of Mcl-1 expression [29]. Mcl-1 transcription has been reported to be regulated by the transcription factors SRF/ETS, STAT3, CREB and PU.1 [38]. The transcription factors involved in TRAIL/ MAPK-dependent modulation of Mcl-1 in NSCLC remains to be identified. In addition, Mcl-1 is also subject to post-translational control, as it is degraded by the 26S proteasome after ubiquitylation by the E3 ligase MULE [39]. Mcl-1 degradation was shown to be regulated through phosphorylation at different sites by ERK, JNK, and GSK-3β, and phosphorylation at Thr163 by ERK prolonged the Mcl-1 half-life [40]. A previous study in hepatocytes showed that JNK phosphorylation of Mcl-1 at Ser121, in conjunction with Thr163, stabilizes Mcl-1 and protects against TNFα-induced apoptosis [41]. Thus, in addition to transcriptional effects, TRAIL-induced JNK and p38 may also lead to posttranslational modification of Mcl-1 in NSCLC cells and affect protein stability. Furthermore, it was reported that the pro-apoptotic Bcl-2 homolog Bim is involved in mediating the TRAIL-dependent enhancement of Fas-induced apoptosis in hepatocytes that occurred through JNK activation [42]. However, in NSCLC cells we did not detect changes in the expression of Bim following TRAIL treatment and inhibition of either JNK or p38 (data not shown).

Based on our findings in H460 cells we propose a model in which TRAIL induces p38 activation in a RIP1–dependent manner causing a reduction in the level of Mcl-1, thus enhancing apoptosis. The activation of caspase-8 leads to RIP1 cleavage that is required for JNK activation subsequently causing enhanced Mcl-1 expression and suppression of apoptosis (see Fig. 7). JNK on its turn appears to suppress RIP1 cleavage in a direct or

indirect way. The precise mechanism by which RIP1 prevents activation of JNK and is involved in the proapoptotic activation of p38 remains elusive.

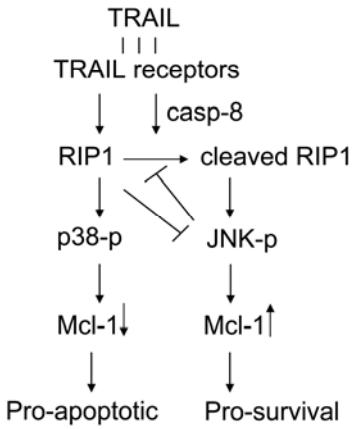

Figure 7. Proposed model for TRAIL-induced p38 and JNK activation leading to pro-apoptotic and pro-survival effects. TRAIL activates the pro-apoptotic activity of p38 in a RIP1-dependent way. JNK activation occurs following cleavage of RIP1 by caspase-8 and JNK on its turn has a suppressive effect on RIP1 cleavage via as yet unknown mechanisms. P38 and JNK affect apoptosis by modulating the expression of their know substrate Mcl-1, with p38 and JNK leading to down- or upregulation of Mcl-1 levels, respectively.

As JNK was found to have a pro-survival effect in TRAIL signaling in NSCLC, it would be interesting to combine TRAIL with JNK inhibitors in this cancer type. Currently, some JNK inhibitors such as the isoquinolones and piperazine amides are in development and combination therapies with TRAIL await further testing [43]. It should be noted that the short JNK1 isoforms were found to be anti-apoptotic, while the long isoforms transmitted a pro-apoptotic signal in response to TRAIL in colon cancer cells [44]. So, the design of JNK isoform selective inhibitors isoform inhibitors should also be taken into consideration. Here, we did not distinguish between the different isoforms of JNK, as SP600125 inhibits the JNK1,-2, and -3 isoforms with similar potency [24].

In conclusion, we found that TRAIL induced the activation of the MAP kinases JNK and p38 in NSCLC cells in which RIP1 played a modulatory role, and cumulating in changes in Mcl-1 expression and subsequent apoptosis activation. In a subset of NSCLC tumors inhibition of JNK may provide a strategy for enhancing TRAIL-induced apoptosis.

Acknowledgements

This research was performed within the framework of project T3-112 of the Dutch Top Institute Pharma and supported by grant RUG2011-5211 from the Dutch Cancer Society.

Conflict of interest

None

Reference List

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60(5):277-300.
2. Blackstock AW, Govindan R. Definitive chemoradiation for the treatment of locally advanced non small-cell lung cancer. J Clin Oncol 2007; 25(26):4146-4152.
3. Lu C, Lee JJ, Komaki R, Herbst RS, Feng L, Evans WK et al. Chemoradiotherapy with or without AE-941 in stage III non-small cell lung cancer: a randomized phase III trial. J Natl Cancer Inst 2010; 102(12):859-865.
4. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39(7-8):1462-1475.
5. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
6. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 2008; 8(10):782-798.
7. Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, Westendorp BF et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 2010; 138(7):2357-2367.
8. Ishimura N, Isomoto H, Bronk SF, Gores GJ. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 2006; 290(1):G129-G136.
9. Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. J Cell Sci 2012.
10. Karacay B, Sanlioglu S, Griffith TS, Sandler A, Bonthius DJ. Inhibition of the NF-kappaB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells. Cancer Gene Ther 2004; 11(10):681-690.
11. Kim YS, Schwabe RF, Qian T, Lemasters JJ, Brenner DA. TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. Hepatology 2002; 36(6):1498-1508.
12. Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D et al. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. Circulation 2003; 107(17):2250-2256.
13. Thiefes A, Wolter S, Mushinski JF, Hoffmann E, ttrich-Breiholz O, Graue N et al. Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. J Biol Chem 2005; 280(30):27728-27741.
14. Vaculova A, Hofmanova J, Soucek K, Kozubik A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. FEBS Lett 2006; 580(28-29):6565-6569.
15. Weldon CB, Parker AP, Patten D, Elliott S, Tang Y, Frigo DE et al. Sensitization of apoptotically-resistant breast carcinoma cells to TNF and TRAIL by inhibition of p38 mitogen-activated protein kinase signaling. Int J Oncol 2004; 24(6):1473-1480.
16. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene 2003; 22(19):2869-2881.
17. Mathews ST, Plaisance EP, Kim T. Imaging systems for westerns: chemiluminescence vs. infrared detection. Methods Mol Biol 2009; 536:499-513.
18. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002; 2(3):243-247.
19. van Leuken R, Clijsters L, van ZW, Lim D, Yao X, Wolthuis RM et al. Polo-like kinase-1 controls Aurora A destruction by activating APC/C-Cdh1. PLoS One 2009; 4(4):e5282.

20. Giovannetti E, Zucali PA, Assaraf YG, Leon LG, Smid K, Alecci C et al. Preclinical emergence of vandetanib as a potent antitumour agent in mesothelioma: molecular mechanisms underlying its synergistic interaction with pemetrexed and carboplatin. Br J Cancer 2011; 105(10):1542-1553.
21. Voortman J, Resende TP, bou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 2007; 6(7):2103-2112.
22. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
23. Krishna M, Narang H. The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell Mol Life Sci 2008; 65(22):3525-3544.
24. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 2001; 98(24):13681-13686.
25. Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D et al. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 1997; 272(18):12116-12121.
26. Wang Z, Canagarajah BJ, Boehm JC, Kassisa S, Cobb MH, Young PR et al. Structural basis of inhibitor selectivity in MAP kinases. Structure 1998; 6(9):1117-1128.
27. Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 2008; 4(5):313-321.
28. Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res 2000; 60(24):7133-7141.
29. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of MCL-1. Cell Death Differ 2010; 17(8):1288-1301.
30. Trouillas M, Saucourt C, Duval D, Gauthereau X, Thibault C, Dembele D et al. Bcl2, a transcriptional target of p38alpha, is critical for neuronal commitment of mouse embryonic stem cells. Cell Death Differ 2008; 15(9):1450-1459.
31. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
32. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005; 12(3):228-237.
33. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 2011; 75(1):50-83.
34. Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ 2007; 14(3):400-410.
35. Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 1999; 13(19):2514-2526.
36. Martinon F, Holler N, Richard C, Tschopp J. Activation of a pro-apoptotic amplification loop through inhibition of NF-kappaB-dependent survival signals by caspase-mediated inactivation of RIP. FEBS Lett 2000; 468(2-3):134-136.
37. Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol 2000; 20(18):6638-6645.
38. Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. FEBS Lett 2010; 584(14):2981-2989.
39. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005; 121(7):1085-1095.

40. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene 2004; 23(31):5301-5315.
41. Kodama Y, Taura K, Miura K, Schnabl B, Osawa Y, Brenner DA. Antiapoptotic effect of c-Jun N-terminal Kinase-1 through Mcl-1 stabilization in TNF-induced hepatocyte apoptosis. Gastroenterology 2009; 136(4):1423-1434.
42. Corazza N, Jakob S, Schaer C, Frese S, Keogh A, Stroka D et al. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. J Clin Invest 2006; 116(9):2493-2499.
43. Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY, Ng DC. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochim Biophys Acta 2010; 1804(3):463-475.
44. Mahalingam D, Keane M, Pirianov G, Mehmet H, Samali A, Szegezdi E. Differential activation of JNK1 isoforms by TRAIL receptors modulate apoptosis of colon cancer cell lines. Br J Cancer 2009; 100(9):1415-1424.

Chapter 4

Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells

Kaamar Azijli, Saravanan Yuvaraj, Maikel P. Peppelenbosch, Thomas Würdinger, Henk Dekker, Jos Joore, Evert van Dijk, Wim J. Quax, Godefridus J. Peters, Steven de Jong, Frank A.E. Kruyt

Journal of Cell Science, 2012; 125, 4651-4661

ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) triggers apoptosis selectively in tumor cells through interaction with TRAIL-R1/DR4 or TRAIL-R2/DR5 and is considered a promising avenue in cancer treatment. TRAIL resistance, however, is frequently encountered and hampers anti-cancer activity. Here we show that whereas H460 non-small cell lung cancer (NSCLC) cells display canonical TRAIL-dependent apoptosis, A549 and SW1573 NSCLC cells are TRAIL resistant and display pro-tumorigenic activity, in particular invasion, following TRAIL treatment. We exploit this situation to contrast TRAIL effects on the kinome of apoptosis-sensitive cells to that of NSCLC cells in which non-canonical effects predominate, employing peptide arrays displaying 1,024 different kinase pseudosubstrates more or less comprehensively covering the human kinome. We observed that failure of a therapeutic response to TRAIL coincides with the activation of a non-canonical TRAIL-induced signaling pathway constituting amongst others of the activation of Src, STAT3, FAK, ERK and Akt. The use of TRAIL-R1 or TRAIL-R2 selective TRAIL variants subsequently showed that this non-canonical migration and invasion is mediated via TRAIL-R2. Short-hairpin-mediated silencing of RIP1 kinase prevented TRAIL-induced Src and STAT3 phosphorylation and reduced TRAIL-induced migration and invasion of A549 cells. Inhibition of Src or STAT3 by shRNA or chemical inhibitors including dasatinib and 5,15-DPP blocked TRAIL-induced invasion. FAK, Akt and ERK were activated in a RIP1-independent way and inhibition of Akt sensitized A549 cells for TRAIL-induced apoptosis. We thus identified RIP1-dependent and -independent non-canonical TRAIL kinase cascades in which Src and Akt are instrumental and could be exploited as co-targets in TRAIL therapy for NSCLC.

Key Words: TRAIL, migration, NSCLC, RIP1, kinome profiling, Src inhibitors

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. NSCLC accounts for 85% of all lung cancer cases. Although several attempts have been made to develop effective treatment strategies to combat lung cancer, still overall 5-year prognosis is less than 15% in NSCLC [2]. Despite recent successes in sub-populations of NSCLC patients using targeted agents, such as inhibitors of epidermal growth factor signaling (EGF) in patients with mutated EGF receptors, new therapies are urgently needed for improving treatment [3]. An attractive approach for the treatment of NSCLC is provided through the TRAIL receptors.

TRAIL receptors are attractive targets as their stimulation selectively mediates apoptosis activation in tumor cells while sparing normal cells [4]. Apoptosis activation is mediated via two of five known TRAIL receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are thought to encode non-functional receptors, also named decoy receptors. A fifth receptor is osteoprotegerin that acts as a soluble receptor. Apoptosis activation occurs upon ligand-induced TRAIL-R1 or TRAIL-R2 clustering leading to the assembly of the death-inducing signaling complex (DISC). In the DISC, Fas-associated protein with death domain (FADD) binds procaspase-8 or -10 in order to produce active caspase-8 subsequently leading to effector caspases activation and apoptosis [4].

Recombinant TRAIL preparations and TRAIL receptor specific agonistic antibodies have been produced that potently eradicate tumor cells of different origins, including NSCLC, and showed efficacy in various preclinical models [5]. Anti-cancer action of TRAIL receptor targeted agents when applied as single agents, however, is frequently hampered by resistance in tumor cells. For example, approximately half of the NSCLC tumor cells are resistant to the apoptosis-inducing effect of TRAIL unless applied in combination with various conventional or biological agents resulting in sensitive cells [5]. Thus, although currently clinical testing of TRAIL receptor targeted agents shows only moderate efficacy, there is optimism that combination strategies with other therapeutics will overcome resistance and increase therapeutic benefit.

In contrast to its apoptosis-inducing activity, TRAIL has been reported to activate non-apoptotic signaling. In resistant tumor cells TRAIL receptors have been reported to activate pro-inflammatory (NF-κB), pro-survival (PI3K/Akt) and proliferation (mitogen activated protein kinases (MAPKs)) pathways [6]. Activation of these non-apoptotic signaling pathways involves the recruitment of a number of proteins to the receptors, such as receptor-interacting protein 1 (RIP1), TNF receptor associated factor 2 (TRAF2), TNF receptor type 1-associated death domain (TRADD) and NEMO/IKK-γ, thus forming secondary intracellular complexes [7;8]. The precise compositions and sequence of formation of the multiprotein complexes in which these factors reside are currently elusive. However, the death domain containing kinase RIP1 was shown to play a pivotal

role in the activation of NF-κB, JNK, p38 and ERK1/2 by TRAIL, and in the activation of necroptosis by TNF [9;10]. More recently TRAIL has also been implicated in the promotion of tumor cell invasion in pancreatic ductal adenocarcinoma and colorectal cancer models [11;12], further illustrating pro-tumorigenic/ non-canonical activity of TRAIL in resistant tumor cells. Clearly, the dual activity of TRAIL can hamper its clinical activity and delineating these unwanted effects is essential for developing strategies to revert them into pro-apoptotic signals. Together, these considerations prompted us for a systematic evaluation of the nature of these non-canonical TRAIL-dependent pathways.
In the present study we have examined the possible pro-tumorigenic effects of TRAIL on NSCLC cells. Particularly we found that TRAIL induces migration and invasion of TRAIL-resistant NSCLC cells. A kinome profiling strategy was used to dissect underlying molecular mechanisms and the identified kinases were further examined for involvement in non-canonical TRAIL signaling. Collectively, our results identified a novel TRAIL-induced Src-STAT3-dependent tumor cell migratory pathway and imply enhanced therapeutic benefit of TRAIL therapy when combined with Src and PI3K/Akt inhibition.

MATERIALS & METHODS

Cell lines and chemicals

NSCLC cells, H460, H322, SW1573, and A549, derived from ATCC in 2003, were cultured as monolayers in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. The cell lines were tested for their authenticity by short tandem repeats (STR) profiling DNA fingerprinting (Baseclear, Leiden, The Netherlands). PP2, PD098059 (Sigma-Aldrich, St. Louis, MO, USA), dasatinib and saracatinib (both from LC Laboratories, Woburn, MA, USA) were dissolved in DMSO to 20 mM stock solutions. LY294002 (Sigma-Aldrich) was dissolved in DMSO to 10 mM stock solution.

MTT assay

A total of 10,000 cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany). The next day 100 µl medium with or without TRAIL was added with increasing concentrations to the cells. After 24 h incubation, the medium was discarded and 50 µl of a MTT solution (0.5 mg/ml (Sigma-Aldrich) in HBSS) was added and incubated at 37°C for 1.5 h. The formazan crystals were dissolved using 150 µl dimethyl sulfoxide (DMSO) and absorbance was measured at 540 nm (Tecan, Männedorf, Switzerland). Results are presented as percentage of viable cells taking the control (untreated cells) as 100% survival. The concentration resulting in 50% of cell growth inhibition (IC_{50}) was derived from the growth inhibition curve.

Receptor cell surface expression

Analysis of TRAIL-receptor membrane expression was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL, USA). Adherent cells were harvested by treatment with trypsin and washed twice in PBS containing 1% BSA. Appropriate concentrations of antibodies dissolved in PBS/1% BSA were added to the cells. The following antibodies were used to determine TRAIL receptor membrane expression: TRAIL-R1 (HS101), TRAIL-R2 (HS201), TRAIL-R3 (HS301), TRAIL-R4 (HS402), all from Alexis. Mouse IgG (DAKO) was used as isotype control. Subsequently, cells were incubated for 30 min on ice, washed twice with cold PBS/1% BSA, and incubated with FITC-conjugated rabbit-antimouse (DAKO, Glostrup, Denmark) for 30 min on ice. After washing, the cells were analyzed by flow cytometry. Surface expression is shown as a ratio of the signal of the specific TRAIL-receptor antibody and the negative isotype control antibody.

Migration assays

Cell migration was determined using the wound healing assay as described previously [13]. In brief, NSCLC cells were seeded in 96-wells plates and grown till confluence. A 96 well floating-pin transfer device with a pin diameter of 1.58 mm coming to a flat point at

the tip with a diameter of 0.4 mm (VP Scientific VP-408FH) was used to make the scratches [14]. TRAIL was added for 15 min and after medium refreshment migration was monitored. Indicated kinase inhibitors were incubated for 30 min, followed by 15 min incubation with TRAIL. Wounds were captured at 2.5x magnification with a microscope (DMIRB, Leica Microsystems, Wetzlar, Germany), and Q500MC software (Leica Microsystems) at 0 and 8 h. The wound width at 8 h was measured in four areas and compared with the initial width at the 0 h time point.

Invasion assays

The invasion assay was carried out by using transwell chambers with fluorescence-blocking 8 µm pore polycarbonate filter inserts (#35-1152; HTS Fluoroblock Insert, Falcon, Becton Dickinson Labware, Bedford, MA) in 24-wells plates [13]. The insert was coated overnight at RT with 100 µl matrigel (50 ng/ml in PBS; Sigma-Aldrich). The cells were treated for 15 min with TRAIL. In each insert 200,000 H460 or A549 cells were seeded in serum free RPMI 1640 medium. For H322 and SW1573 800,000 cells were taken. In the bottom compartment, medium containing 10% FCS was added. NSCLC cells were allowed to invade for 8 h. After 8 h, 5 µM calcein-AM (Molecular Probes, Eugene, OR, USA) was added to the lower compartment for 30 min. Pictures were captured and fluorescently labeled cells were counted.

Kinome profiling arrays

The PepChip® kinome array (Pepscan Systems, Lelystad, the Netherlands) consisting of 1,024 peptides with specific phosphorylation sites was used to evaluate the kinome after TRAIL treatment in NSCLC cells, similarly as described previously [15;16]. Lysates generated from H460 and A549 cells treated with or without TRAIL (15 min) were analyzed in the assays.

PepChip® data analysis

A PepChip® contains 1,024 peptides that are spotted in triplicate. Two slides were taken for one condition and experiments were performed in duplicate, resulting in 12 data points per condition. The spots were quantified using the ScanAlyze software and the mean intensity of the 12 data points representing a specific peptide was calculated. Spots deviating more than 2× standard-deviation were excluded. Peptides were considered to represent true phosphorylation events when the average phosphorylation minus 1.96 times the standard deviation of the 12 spots was higher than the value expected from the background distribution. A value of 1.96 was taken, which yields a p value of at least 0.05. A list of peptides was generated by ranking-ordering the spots and curve-fitting analysis, resulting in an “On” or “Off”-call for each peptide used to create provisional signal transduction schemes as described earlier [17].

Western blotting

Western blot analysis was performed as described before [13]. The following primary antibodies were used all from Cell Signalling Technology Inc. (Danvers, MA, USA): anti-p-Src (Tyr416) (#2101), anti-Src (#2109), anti-p-Akt (Ser473) (#9271), anti-Akt (#9272), anti-p-ERK 42/44 (Thr202/Tyr204) (#9101), anti-ERK (#9102), anti-p-STAT3 (Ser727) (#9134), anti-STAT3 (#9132), and RIP (#3493). β-Actin was from Sigma-Aldrich. The bands were analyzed and the activities of the kinases were determined by calculating the ratio between the phosphorylated form and the total kinase.

Gene silencing

For silencing the expression of genes pSUPER.retro was used similarly as described previously [18]. Targeted short hairpin (sh)RNA sequences were inserted into the BglII and HindIII sites of the pSUPER.retro vector. All cloned shRNA sequences were verified by DNA sequencing. Retroviruses were packaged and introduced into cells as described previously [19]. A549 and H460 cells were retrovirally infected with control pSUPER.retro or pSUPER.retro-shRIP1 (RIP1-targeting sequence #1, 5'- GAGCAGCAGTTGATAATGT-3'; RIP1-targeting sequence #2 5'- TACCACTAGTCTGACGGATAA-3') or pSUPER.retro-shSrc (5'-GGACCTTCCTCGTGCGAGA-3') for 24 h. Infected cells were selected with 2 µg/ml puromycin.

Cell death measurement

Cell death measurements were performed by FACS analysis as described previously [20]. In brief, cells were seeded at a density of 400,000 cells/well in 6-wells plates. After treatment, cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1,200 rpm. Subsequently, cells were stained with propidium iodide (Sigma-Aldrich) buffer (0.1 mg/ml with 0.1 % RNAse A (Qiagen, Venlo, the Netherlands) in dark on ice. DNA content of the cells was analyzed by FACS (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G_1 peak was used to determine the extent of cell death.

RESULTS

TRAIL induces a spectrum from pro-apoptotic to pro-invasive responses in different NSCLC cells

TRAIL resistance of cancer cells hampers therapeutic exploitation of the TRAIL apoptotic pathway and may even provoke counterproductive effects. Also *in vitro*, different NSCLC cell lines differ markedly in their sensitivity to TRAIL as judged by the capacity of a 24 h TRAIL treatment to diminish cell viability as measured in MTT assays (Fig. 1A and B). Whereas H460 cells are highly sensitive for TRAIL (IC_{50} ≈ 10 ng/ml), H322 cells require higher concentrations to respond with cell death to TRAIL treatment (IC_{50} ≈ 81 ng/ml), and SW1573 and A549 cells seem incapable of inducing an apoptotic response to TRAIL (IC_{50} > 200 ng/ml).

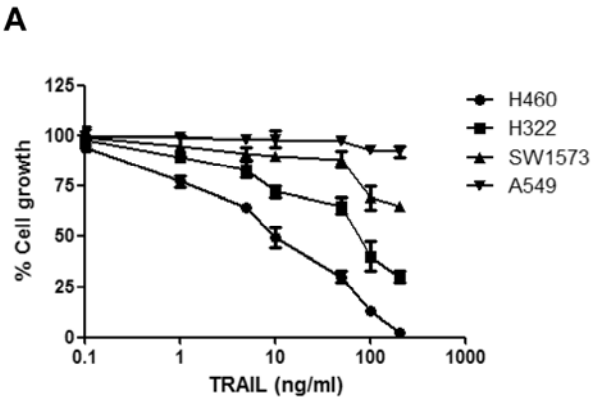

Figure 1. Cytotoxic effects of TRAIL in different NSCLC cell lines. (**A**) Cell growth was measured using an MTT assay following 24 h treatment with increasing concentrations of TRAIL. (**B**) calculated IC_{50} values for TRAIL in the different NSCLC cells.

Importantly, resistance to cell death is associated with TRAIL-induced pro-invasive effects in NSCLC. In wound healing assays, TRAIL treatment resulted in 2 to 3-fold increased migratory activity of A549 cells when compared to untreated cells (Fig. 2A and B). Also, SW1573 cells showed an increase in migration activity in this assay upon TRAIL challenge, whereas TRAIL failed to induce migration in H322 and H460 cells in line with apoptosis activation in these cells. MTT assays performed in parallel showed that the enhanced 'wound healing' in A549 cells is not due to a TRAIL-mediated increase in proliferation (Fig. 2E). Consistently, a decreased potential to respond to TRAIL stimulation with apoptosis is associated with increased invasion as detected in matrigel-transwell assays. TRAIL apoptosis-resistant A549 cells exhibited approximately a 2-fold increase in invasive capacity following TRAIL stimulation when compared to untreated cells (Fig. 2C and D).

Accordingly, SW1573 displayed enhanced invasion following TRAIL treatment, whereas H322 cells were not affected and H460 cells showed reduced invasion - correlating with the pro-apoptotic activity of TRAIL. We conclude that using this panel of NSCLC cell lines we can study a spectrum of TRAIL responses, ranging from full therapeutic sensitivity (H460 cells) to a cell line that almost exclusively displays non-canonical pro-oncogenic effects following TRAIL treatment (A549 cells), and we decided to exploit this situation to obtain fundamental insight into the molecular basis of the differential TRAIL response. Experiments were therefore initiated to characterize the signal transduction events involved.

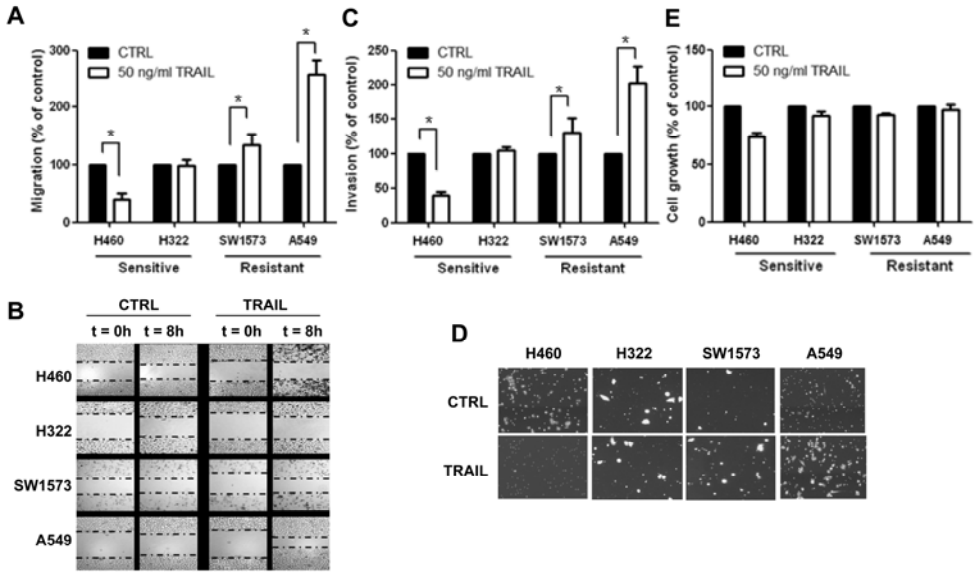

Figure 2. TRAIL induces migration and invasion in resistant NSCLC cells. (**A**) migration in H460, H322, SW1573, and A549 cells treated for 15 min with TRAIL. (**B**) the wound closure was measured after 8 h, as shown in a representative wound healing experiment. (**C**) invasive effect of TRAIL on the NSCLC cell lines assessed with (**D**) matrigel-transwell assays as shown in a representative experiment. E, cell growth determined by MTT assays of cells treated with 50 ng/ml TRAIL for 15 min and further culturing for 8 h with refreshed medium.

Kinome profiling of TRAIL signaling in resistant and sensitive cells

The apparent dichotomy between canonical pro-apoptotic TRAIL responses in H460 cells and non-canonical pro-invasive TRAIL responses in A549 cells allowed us to delineate the signal transduction elements involved in TRAIL signaling. We resorted to using kinome profiling employing peptide arrays exhibiting 960 different kinase pseudosubstrates that provide comprehensive descriptions of the cellular kinome. This allowed us to contrast TRAIL-induced kinomic changes in the canonically signaling H460 cells to those in the non-canonical signaling A549 cells. To this end, cells were treated for 15 min either with

vehicle control or with 50 ng/ml TRAIL in order to detect early kinase activation events. The obtained kinome profiles of treated and untreated cells of each cell line revealed substantial differences between TRAIL-induced kinase activity in A549 and H460 cells (for a selection see Table 1). Importantly, a signal transduction cascade emerged in which A549 cells respond to TRAIL by activation of a proximal tyrosine kinase activity associated with several signaling events like increased JAK2 signaling to STAT3 and enhanced PI3K activity led to activation of the Akt/mTOR/S6 kinase signaling pathway. There is also increased activity of Rac, Rho and Src signaling able to enhance FAK activity and phosphorylation to other cytoskeleton remodeling proteins (*e.g.* cortactin, vimentin; see Table 1) and increased activity of the Raf/MEK/ERK signaling cassette.

Table 1 | Effects of TRAIL stimulation on kinase activity in TRAIL-sensitive H460 cells and TRAIL-resistant A549 cells.

peptide used for kinase reaction	phosphorylation site in protein	upstream kinase	Ratio H460	Ratio A549
KTRDQ**Y**LMWLT	p85-PI3Kα $_{Tyr580}$	TyrK	1.25	1.67*
LTIDR**Y**LAIVH	CCR2$_{Tyr139}$	JAK2	0.97	1.31*
GSAAP**Y**LKTKF	STAT3$_{Tyr705}$	JAK2/Src	1.09	1.75*
TEDQY**S**LVEDD	p85-PI3Kα $_{Ser608}$	p85-PI3Kα	0.88	14.64*
RLRTH**S**IESSG	MEK2$_{Ser80}$	Akt	0.68	1.29**
RPRSC**T**WPLPR	FKHR$_{Thr24}$	Akt	0.50	1.59*
RRRAA**S**MDSSS	AFX$_{Ser196}$	Akt	0.69	1.79**
RSRHS**S**YPAGT	Bcl2$_{Ser72}$	Akt	0.90	1.37**
ELRRM**S**DEFVD	Bcl2$_{Ser118}$	Akt	0.71	1.40*
RLRPL**S**YPQTV	Rac1	Akt	0.60	1.89*·
RTRTD**S**YSAGQ	mTor$_{Ser2448}$	mTor	1.00	1.50*
RRRMA**S**MQRTG	p300EBP$_{Ser1834}$	p70S6K	1.77	1.52*
RRGDS**Y**DLKDF	Vav1$_{Tyr441}$	?	0.63	1.75***
GGDDI**Y**EDIIK	Vav2$_{Tyr172}$	?	0.88	2.82***
ASRPS**S**SRSYV	Vimentin$_{Ser26}$	PAK	0.67	1.79*
AEDST**Y**DEYEN	Cortactin$_{Tyr486}$	Src	0.54	2.30**
NVVPL**Y**DLLLE	Estrogen Receptor α	Src	1.16	1.42**
EDSTY**Y**KASKG	FAK$_{Tyr577}$	Src	1.50	1.29*
IESDI**Y**AEIPD	FAK2$_{Tyr402}$	FAK	1.13	1.71*
MEDYD**Y**VHLQG	p130Cas$_{Tyr666}$	FAK	0.63	2.44***
KTNLS**Y**YEYDK	BMX$_{Tyr40}$	FAK	1.31*	1.84*
IPRRT**T**QRIVA	CRMP2$_{Thr555}$	ROCK	0.60	2.58***
RDKYK**T**LRQIR	Moesin$_{Thr564}$	ROCK	1.25	2.72*
INRSA**S**EPSLH	Raf1$_{Ser621}$	Raf1	0.77	1.46*
DSMAN**S**FVGTR	MEK1$_{Ser222}$	Raf1	0.51	1.89**
VKRRP**S**PYEME	TAL1$_{Ser172}$	ERK	0.75	2.25***
PPVPA**T**PYEAF	SPIB$_{Thr56}$	ERK	1.25	1.74*
LKGPG**T**PAFPH	C/EBPε$_{Thr74}$	ERK	1.37*	1.17*
KVEPA**S**PPYY	PPARγ$_{Ser112}$	ERK	0.94	1.58*
NLLPM**S**PEEFD	STAT$_{Ser727}$	ERK	0.65	1.49*

Peptide arrays were used on the selected signal-transduction-relevant undecapeptide kinase substrates listed. Values are ratios (15 min TRAIL/control) of STORM phosphoimager units (24 h exposure) of 12 replicates. *P,0.05; **P,0.01; ***P,0.001.

Non-canonical TRAIL-dependent kinase activities are reflected in altered levels of target protein phosphorylation

The kinome profiling assay measures kinase activities, but within a cell kinase activities are counterbalanced by the enzymatic activity of phosphatases. Hence it is important to establish if the differences in kinase activities as determined by kinome profiling are reflected in altered levels of phosphorylation of target proteins. In accordance with the PepChip results, Western blot experiments revealed that already within 5 min after TRAIL treatment Src and ERK were phosphorylated, whereas Akt, STAT3 and FAK phoshorylation were detectable at 10 to 15 min post-treatment in A549 cells (Fig. 3A). However, not all identified possible targets or upstream kinases could be corroborated. For example, TRAIL-induced VAV2 and JAK2 phosphorylation was not observed by Western Blotting, while we did not find evidence of activation of ROCK (not shown). Basal and TRAIL-induced phosphorylation of a number of kinases, including Src, Akt, ERK, FAK and STAT3, was variable in the other cell lines (Fig. 3B). In TRAIL resistant SW1573 cells increases in phosphorylation of Src ERK, and FAK by TRAIL were seen. In H460 no detectable effects were observed and in intermediate TRAIL sensitive H322 cells, ERK phosphorylation was slightly elevated after TRAIL exposure (Fig. 3B). Thus the non-canonical TRAIL signaling pathway identified by kinome profiling is reflected in meaningful changes in target protein phosphorylation.

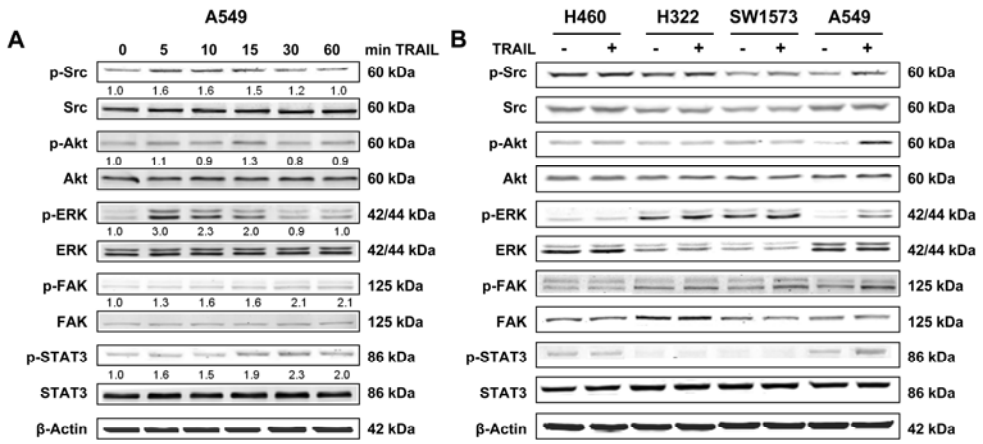

Figure 3. TRAIL-induced kinase activation in NSCLC cells; confirming kinases identified by PepChip kinase arrays. (**A**) the indicated kinases were evaluated for TRAIL-induced phosphorylation during time by Western blotting in A549 cells using antibodies detecting phosphorylated and total kinase. Numbers represent the quantified bands as ratio of the intensity of bands for the phosphorylated form: band of the total protein. The untreated control was set at 1.0. (**B**) kinase activation in H460, H322, SW1573, and A549 cells for 15 min after treatment with 50 ng/ml TRAIL determined by Western blotting.

Non-canonical TRAIL responses are mediated through the TRAIL-R2

Earlier we generated TRAIL variants that selectively engage either TRAIL-R1 or TRAIL-R2, named 4C7 and DHER respectively [21;22]. We found these variants to be more potent than wild-type TRAIL in triggering apoptosis in a tumor cell line-dependent fashion. We now employed these variants to determine the TRAIL receptor mainly responsible for the induction of non-canonical pro-invasive properties of TRAIL. A549 cells express both TRAIL-R1 and –R2, whereas decoy receptors are hardly detectable on the cell surface (Fig. 4A), in agreement with our previous findings [23]. As shown in Fig. 4B and C, migration and invasion of A549 cells is enhanced when stimulated with the TRAIL-R2 selective ligand (DHER), and not by TRAIL-R1 selective 4C7. Thus, the non-canonical migratory and invasive features of TRAIL in resistant NSCLC cells are predominantly mediated by TRAIL-R2. Furthermore, comparing DHER- with TRAIL-treated A549 cells showed a similar kinase phosphorylation profile (Fig. 4D).

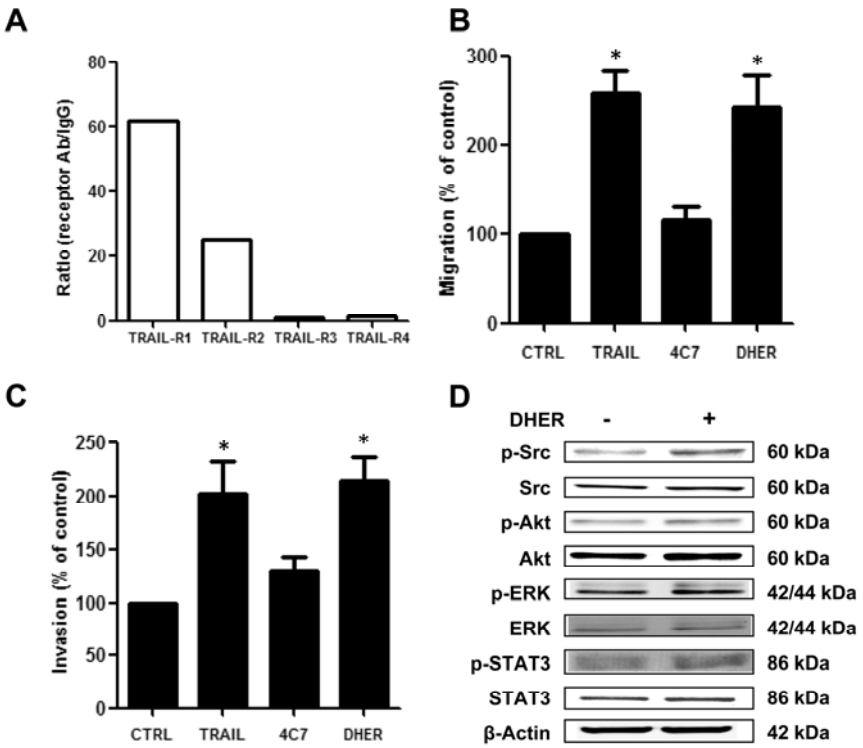

Figure 4. TRAIL induces migration and invasion in A549 cells mainly via TRAIL-R2. (**A**) TRAIL receptor surface expression in A549 cells determined by FACS analysis. (**B**) effect of TRAIL-R1 (4C7) and TRAIL-R2 (DHER) specific TRAIL variants on migration, and (**C**) invasion are shown. Levels of untreated cells were set at 100%. The averages of experimental triplicates (±s.d.) are shown. *p<0.05, t test compared to CTRL. (**D**) Western blots showing phosphorylation status of indicated kinases in A549 cells after 15 min treatment with 50 ng/ml DHER.

The migratory and invasive effects of TRAIL are mediated by RIP1 and involves activation of Src

We next examined the role of RIP1 kinase in signaling pathways that mediate migration and invasion. RIP1, part of the non-apoptotic signaling complex, is known to be involved in TRAIL-induced kinase activation although its precise role is not well understood [8;24]. RIP1 was silenced using a selective shRNA in A549 and H460 cells and knockdown was confirmed by Western blotting (Fig. 5A).

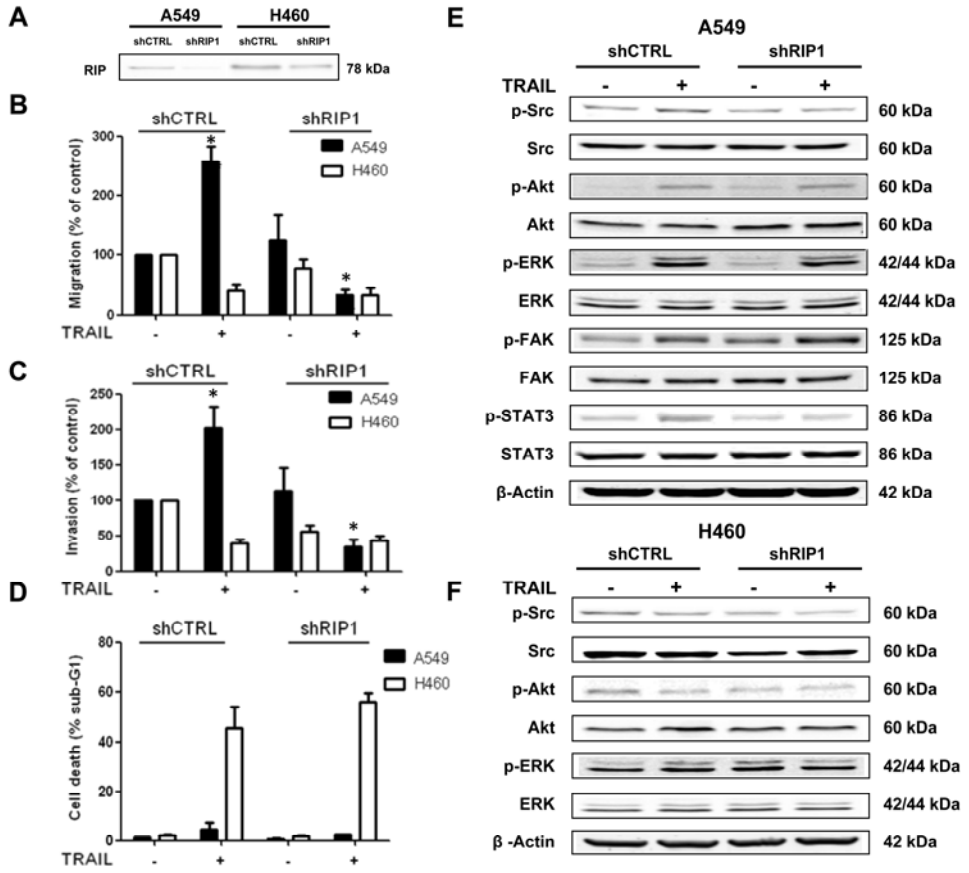

Figure 5. TRAIL-induced migration and invasion, and Src-STAT3 activation is RIP1 dependent. (**A**) expression of RIP1 was effectively silenced by a specific shRNA in H460 and A549 cells as determined by Western blotting. (**B**) TRAIL-dependent cell migration was determined in wound healing assays, and (**C**) invasion in matrigel-transwell assays. (**D**) effect of RIP1 knockdown on TRAIL-induced apoptosis in A549 and H460 cells. (**E**) Western blots showing the effect of RIP1 knockdown on TRAIL-dependent kinase activation (50 ng/ml TRAIL for 15 min) in A549 cells and (**F**) in H460 cells. The averages of experimental triplicates ±s.d. are shown. *p<0.05, t test, compared to each other.

A549-shRIP1 cells clearly showed a decrease in TRAIL-dependent migration in wound healing assays (Fig. 5B). The invasive effect of TRAIL was also abrogated in the absence of RIP1 (Fig. 5C). In contrast, there were no differences in TRAIL-dependent migration and invasion in H460-shRIP1 cells compared to control cells. RIP1 knockdown in A549 cells did not result in a detectable increase in TRAIL sensitivity (Fig. 5D).
The involvement of RIP1 in TRAIL-induced kinases activation was examined in more detail. As depicted in Fig. 5E, TRAIL-induced Src and STAT3 phosphorylation is abrogated in A549-shRIP1 cells. However, RIP1 depletion did not affect the phosphorylaton of Akt, ERK, and FAK following TRAIL treatment. In H460 cells phosphorylation of Src, Akt and ERK was not significantly altered (Fig. 5F). The abrogation of TRAIL-induced migration/ invasion in A549-shRIP1 cells together with a lack of Src and STAT3 activation is suggestive of a role of these kinases in controlling the metastasis-prone events.

TRAIL-dependent activation of a RIP1-Src-STAT3 cascade mediates invasive behavior
To further investigate the role of Src in mediating TRAIL-induced migration/invasion, Src expression was silenced with shRNA in A549 cells. In these cells TRAIL-induced migration and invasion was completely inhibited (Fig. 6A and 6B). Knockdown of Src did not result in an increase in cell death after TRAIL treatment when compared to the empty vector control (Fig. 6C). Thus, the observed decrease in migration and invasion was not a consequence of apoptosis induction. Furthermore, three different Src inhibitors, PP2, dasatinib, and saracatinib were employed at concentrations reported to be optimal for selective inhibition of Src as described previously by others [25;26]. As shown in Fig. 6D and E, migration and invasion by TRAIL was completely repressed with each of the Src inhibitors. In addition, these inhibitors particularly when applied at a higher concentration did to some extent enhance TRAIL-induced apoptosis in A549 cells, most notably dasatinib (Fig. 6F). In SW1573 cells, Src inhibition by PP2 also repressed the migratory and invasive effects of TRAIL without significantly affecting apoptosis (Fig. 6G, H and I).

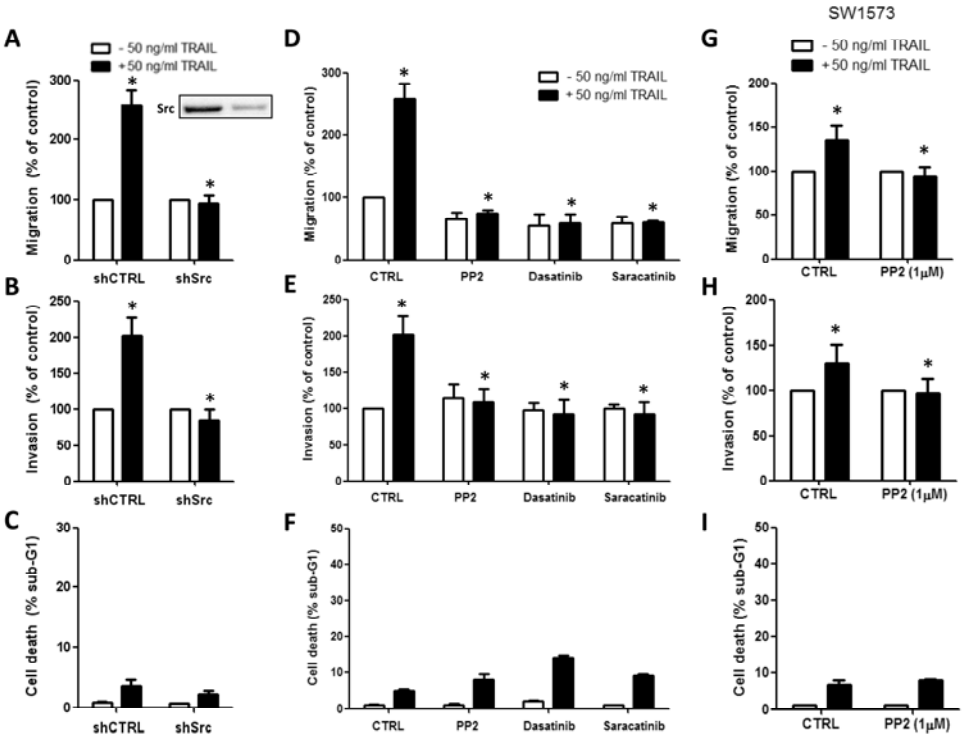

Figure 6. Inhibition of Src blocks migration/ invasion of TRAIL in A549 and SW1573 cells. (**A**) Src knockdown using specific shRNA (confirmed by Western blot in insert) blocks the migratory effect of TRAIL in wound healing assays. (**B**) Src knockdown inhibits invasion by TRAIL in matrigel-transwell chamber assays. (**C**) Silenced Src does not affect TRAIL resistance in A549 cells. A549 cells were incubated with or without chemical inhibitors of Src PP2 (1 μM), Dasatinib (0.01 μM) or Saracatinib (1 μM), to determine effects on migration (**D**) invasion (**E**) and cell death (**F**) following treatment with 50 ng/ml. In SW1573 cells PP2 (1 μM) blocks TRAIL-induced migration (**G**) and inhibits the invasive effect of TRAIL (50 ng/ml) (H). PP2 (1 μM) does not sensitize for TRAIL-induced apoptosis (**I**) Averages of three independent experiments (± s.d.) are shown. *p<0.05, t test, compared to 50 ng/ml TRAIL.

Further delineation of the pathway in A549 cells, using the STAT3 inhibitor 5,15-DPP and the RIP1 inhibitor necrostatin-1, showed attenuation of the migrating and invasive effects of TRAIL (Fig. 7A and B). Necrostatin-1 did not affect apoptosis sensitivity in resistant A549 cells (Fig. 7C), which is in line with shRIP1 knockdown results. Together these results implicate a TRAIL–induced RIP1-Src-STAT3 cascade to drive A549 NSCLC cell invasion.

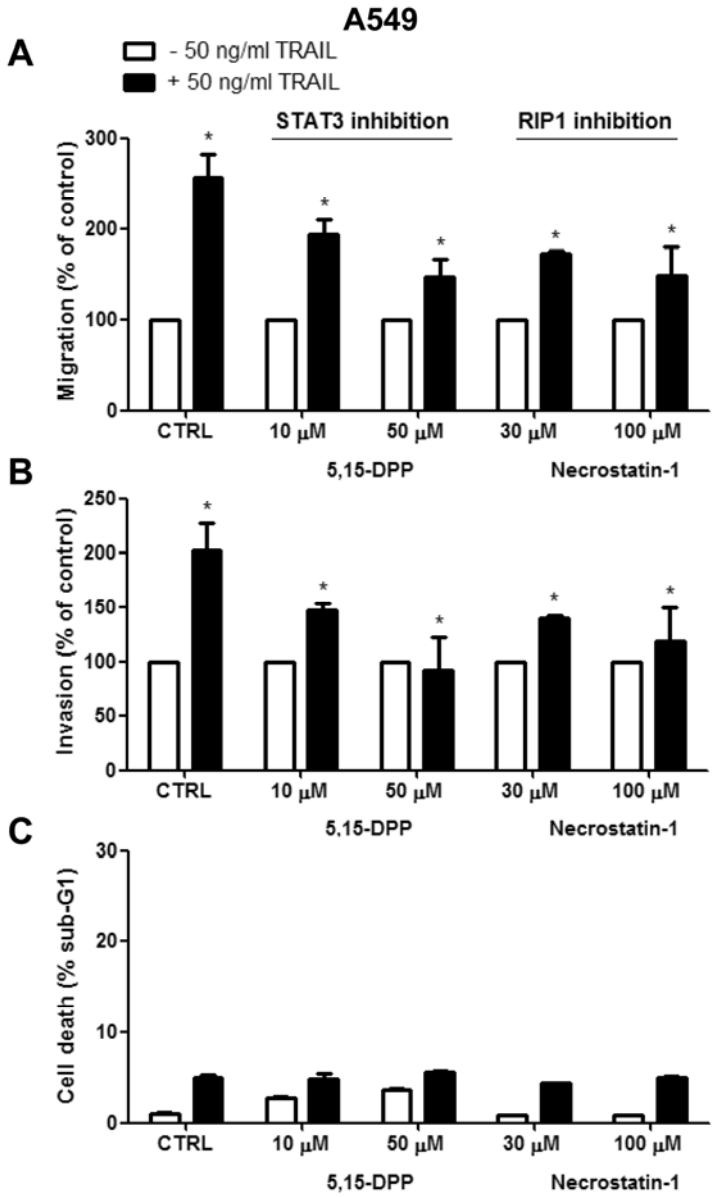

Figure 7. Inhibition of STAT3 and RIP1 blocks migration/invasion of TRAIL in A549 cells. (**A**) A549 cells were incubated with or without chemical inhibitors of STAT3 (5,15-DPP) or RIP1 (necrostatin-1) and with or without TRAIL (50 ng/ml). Migration was determined after 8 h with the wound healing assay. Furthermore, invasion after 8 h (**B**) and cell death after 24 h (**C**) incubation were determined. Averages of three independent experiments (± s.d.) are shown. *p<0.05, t test, compared to 50 ng/ml TRAIL.

TRAIL-induced kinases activation and effects on migration, invasion and apoptosis

Finally, we explored possible interactions between the identified TRAIL-induced kinases cascades. Knockdown of Src in A549 cells prevented, and even decreased, the phosphorylation of STAT3 after TRAIL application. Also TRAIL-induced FAK phosphorylation appeared to depend on Src, whereas p-Akt and p-ERK levels were not affected in A549-shSrc cells (Fig. 8A). On the other hand, inhibition of Akt by LY294002 and ERK by PD098059 also reduced migration and invasion induced by TRAIL (Fig. 8B). Interestingly, inhibition of Akt converted TRAIL resistant A549 cells into apoptosis sensitive cells. In conclusion, we identified novel parallel branches of TRAIL-induced kinase activation that mediate metastases-prone and/ or pro-survival effects (see also Fig. 8C).

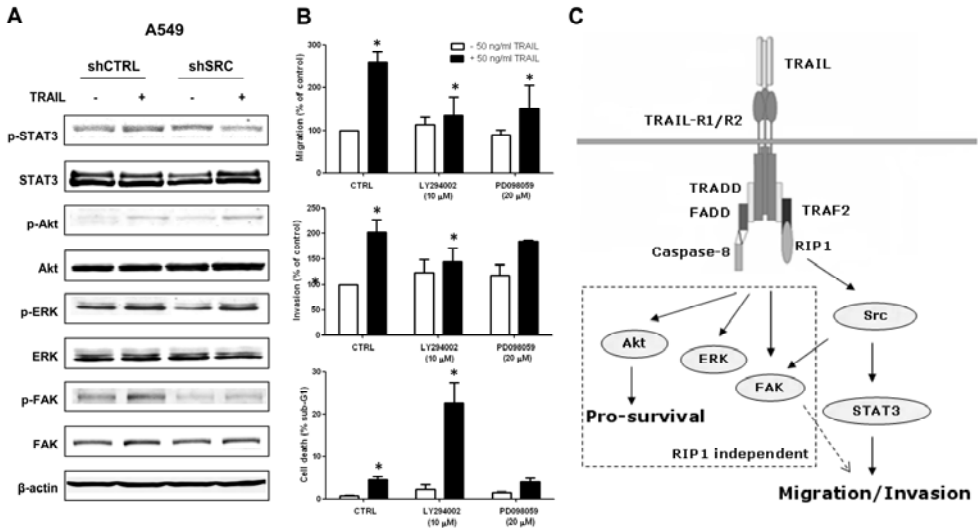

Figure 8. The role of Src in TRAIL-induced kinase activation and effect of Akt and ERK inhibition on migration, invasion and cell death. (**A**) Western blots showing effect of Src knockdown on TRAIL-dependent activation of the indicated kinases. (**B**) Migration, invasion (at 8 h post-treatment) and cell death (24 h after treatment) in A549 cells with or without PI3K/Akt inhibition by LY294002 and ERK inhibition by PD098059. *p<0.05, t test, compared to 50 ng/ml TRAIL. (**C**) Schematic representation of the identified kinases involved in non-canonical TRAIL signaling. RIP1 dependent and independent mechanisms were found leading to parallel activation of pro-survival and migration/ invasion promoting pathways.

DISCUSSION

The TRAIL receptor pathway is currently therapeutically exploited for selective activation of apoptosis in tumor cells. However, stimulation of the TRAIL receptors in preclinical models can also activate unwanted non-apoptotic/non-canonical signaling leading to proliferative, pro-survival and even pro-invasive effects. In the present study using a panel of TRAIL sensitive and resistant NSCLC cell lines we demonstrated non-canonical signaling in resistant cells that enhanced their migratory and invasive properties. Interestingly, whereas the NSCLC cells express both TRAIL-R1 and TRAIL-R2 on their cell surface we only found TRAIL-R2/DR5 to mediate these effects, thus illustrating that the TRAIL receptors have different signaling properties. Intriguing in this respect is an earlier described correlation of high TRAIL-R2 levels in advanced stage NSCLC patients with increased risk of death [27]. The underlying causes of differential signaling are still elusive and are subject of further studies.

Using peptide arrays exhibiting 1,024 specific consensus sequences for protein kinases we identified substrates and corresponding kinases that are stimulated following TRAIL exposure in A549 cells contrasting findings in sensitive H460 cells. Subsequent confirmation of kinase phosphorylation by Western blotting allowed us to identify several kinases that are activated by TRAIL in resistant A549 cells (see also Fig. 8C for a schematic overview). In particular activation of the Src-STAT3 axis was important for mediating TRAIL-induced migration and invasion. The non-receptor protein tyrosine kinase Src is a known activator of signal transduction pathways controlling various key cellular processes such as cell division, proliferation, survival and motility [28]. Elevated Src protein levels and/or kinase activity have been reported in 50-80% of lung cancer cases and correlates with poor patient survival [29]. Src is a known mediator of tumor cell migration and invasion as it leads to reorganization of the cytoskeleton and modulation of the cell adhesion system [30]. Downstream of Src we identified STAT3 as a mediator of migration. This is not unprecedented since recently Src-induced STAT3 activation has been implicated in the formation of podosome structures in primary murine cells facilitating cell migration [31]. STAT3 inhibitors have also been reported to inhibit the migration of prostate cancer cells [32]. Mechanisms described by which STAT3 can promote cell migration include binding of STAT3 to βPIX leading to Rac1 activation [33] and direct phosphorylation of fascin, an actin-bundling protein, by STAT3 [34]. The precise downstream effectors of STAT3 signaling in TRAIL-induced migration/ invasion in NSCLC cells remains to be explored. Another mechanism through which Src can stimulate invasion is via the activation of epithelial-mesenchymal transition (EMT) [35]. The acquisition of mesenchymal properties by epithelial cells characterized by for example loss of E-cadherin and gain of fibronectin expression has been found to stimulate tumor cell dispersion [36]. However, we did not detect clear changes in the expression of these markers in response to TRAIL in NSCLC cells (not shown). Other Src-dependent signals

that have been implicated in migration include activation of the Rho/ROCK/LIMK, Ras/MAPK, PI3K/Akt, FAK/paxillin/CAS pathways [30]. Although the kinome analyses identified possible ROCK activity after TRAIL exposure, we were not able to confirm ROCK activation in Western blots and co-treatment with a ROCK inhibitor did not prevent TRAIL-induced invasion (not shown). On the other hand, knockdown of Src in A549 cells inhibited TRAIL-dependent FAK phosphorylation suggesting a possible role of this pathway as well. FAK is a non-receptor protein tyrosine kinase known to stimulate migration/ invasion when in complex with Src [37]. Of note, in SW1573 cells TRAIL resulted in Src-dependent FAK phosphorylation, but not STAT3 phosphorylation. This may be related to the smaller stimulatory effect of TRAIL on migration/ invasion in SW1573 cells (1.5 fold) when compared to A549 cells (2-3 fold). Inhibition of ERK did partially suppress invasive behavior of A549 cells however TRAIL-induced ERK phosphorylation was independent of Src as was also the case for Akt activation. Active Src has the ability to phosphorylate caspase-8 at tyrosine 380 resulting in blocking of its pro-apoptotic function [38]. However, we could not detect phosphorylated procaspase-8 (not shown) and shRNA-mediated knockdown or chemical inhibition of Src by dasatinib and saracatinib did prevent TRAIL-induced invasion, but failed to effectively sensitize for apoptosis in A549 cells, pointing to other mechanisms of resistance.

TRAIL stimulatory effects on tumor cell migration and invasion have been previously reported to be mediated by NF-κB in apoptosis resistant cholangiocarcinoma cancer cells [39], and by TRAIL-dependent up-regulation of interleukin-8 and chemoattractant protein 1 in pancreatic ductal adenocarcinoma cells [12]. A more recent study has shown that TRAIL-induced migration in colon cancer cells involves oncogenic K-Ras and Raf-1 that convert death receptors into invasion-inducing receptors by suppressing the ROCK/LIM kinase/cofilin pathway [11]. However, the K-Ras status not always predicts the outcome of death receptor signaling, since for example H460 NSCLC cells in our study are highly sensitive for TRAIL-induced apoptosis despite the presence of oncogenic K-Ras. Thus, in NSCLC cells TRAIL-induced Src activation appears to be the main route responsible for migration and invasion.

RIP1 is a serine threonine kinase belonging to the RIP family involved in promoting pro-survival, inflammatory and pro-apoptotic signals depending on the signal and tumor type [10]. RIP1 is part of the secondary complex and has been associated with non-apoptotic functions of TRAIL [8]. To understand the role of RIP1 in our model shRNA-mediated knockdown as well as RIP1 inhibition by necrostatin-1 revealed prevention of TRAIL-induced Src-STAT3 activation and migration/ invasion, corroborating the importance of this pathway. In contrast to a previous report we did not observe sensitization for TRAIL-induced apoptosis in A549 RIP1 knockdown cells [40]. Regarding Akt activation, in prostate adenocarcinoma cells TRAIL-dependent Src activation has been reported to result in activation of PI3K-Akt signaling [41]. We found in NSCLC cells TRAIL to activate a

Src-independent mechanism responsible for Akt activation, and moreover, Akt and also ERK activation was independent of RIP1. The activation of Akt and ERK may be a more indirect consequence of TRAIL pathway activation. For example, in colorectal cancer cells TRAIL was found to activate EGFR and HER2 through Src family kinases (SFK) that in turn activated the cell surface protein A Disintegrin And Metalloproteinase-17 (ADAM-17) also known as Tumor Necrosis Factor Converting Enzyme (TACE) leading to cleavage and shedding of TGF-α. Subsequently, TGF-α activated the EGFR/HER2 pro-survival signaling pathways in an autocrine and paracrine manner [42]. Furthermore, we found TRAIL to be able to phosphorylate FAK in RIP1 knockdown NSCLC cells, in which TRAIL-induced Src activation was prevented. Thus, FAK can be activated in a RIP1-dependent and -independent way, indicating more complex interactions at the level of RIP1, Src and FAK. In this study we identified Akt as an important mediator of TRAIL resistance in NSCLC cells. This confirms earlier studies where the PI3K inhibitors Wortmannin or LY-294002 and the Akt-inhibitor perifosine were shown to cooperate with rhTRAIL to induce apoptosis in NSCLC cells involving increased TRAIL-R2 expression and a reduction of c-FLIP levels [43;44].

Taken together, it can be concluded from our work that TRAIL non-canonical signaling in NSCLC involves a parallel activation of RIP1-dependent and -independent mechanisms that stimulate both pro-survival (Akt) and migration/ invasion (Src, STAT3) mechanisms. Although more work is required to evaluate the unwanted non-canonical effects in other models, our findings may indicate that care should be taken when using TRAIL receptor targeting agents for treating patients. Moreover, treatments with TRAIL agonistic agents in NSCLC may benefit from combined treatment with PI3K/Akt and Src inhibitors in order to potentiate anti-tumor activity and to prevent unwanted side effects.

Acknowledgements

The authors would like to thank dr. Elisa Giovannetti for helpful discussions, Meyram Cil for technical assistance and Ingrid van Roosmalen for the receptor expression experiments. This research was performed within the framework of project T3-112 and T3-103 of the Dutch Top Institute Pharma.

Conflict of interest

Wim Quax receives commercial research support for valorization (STW valorization grant GBC.7660)

Reference List

1. Field JK, Duffy SW. Lung cancer screening: the way forward. Br J Cancer 2008; 99(4):557-562.
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60(5):277-300.
3. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359(13):1367-1380.
4. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
5. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
6. Newsom-Davis T, Prieske S, Walczak H. Is TRAIL the holy grail of cancer therapy? Apoptosis 2009; 14(4):607-623.
7. Jin Z, El-Deiry WS. Distinct signaling pathways in T. Mol Cell Biol 2006; 26(21):8136-8148.
8. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
9. Declercq W, Vanden BT, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. Cell 2009; 138(2):229-232.
10. Meylan E, Tschopp J. The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 2005; 30(3):151-159.
11. Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, Westendorp BF et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 2010; 138(7):2357-2367.
12. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006; 25(56):7434-7439.
13. Bijnsdorp IV, Capriotti F, Kruyt FA, Losekoot N, Fukushima M, Griffioen AW et al. Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors. Br J Cancer 2011; 104(7):1185-1192.
14. Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. BMC Biotechnol 2004; 4:21.
15. Parikh K, Peppelenbosch MP, Ritsema T. Kinome profiling using peptide arrays in eukaryotic cells. Methods Mol Biol 2009; 527:269-80, x.
16. van Baal JW, Diks SH, Wanders RJ, Rygiel AM, Milano F, Joore J et al. Comparison of kinome profiles of Barrett's esophagus with normal squamous esophagus and normal gastric cardia. Cancer Res 2006; 66(24):11605-11612.
17. Fuhler GM, Diks SH, Peppelenbosch MP, Kerr WG. Widespread deregulation of phosphorylation-based signaling pathways in multiple myeloma cells: opportunities for therapeutic intervention. Mol Med 2011; 17(7-8):790-798.
18. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002; 2(3):243-247.
19. van Leuken R, Clijsters L, van ZW, Lim D, Yao X, Wolthuis RM et al. Polo-like kinase-1 controls Aurora A destruction by activating APC/C-Cdh1. PLoS One 2009; 4(4):e5282.
20. Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. Clin Cancer Res 2003; 9(6):2316-2326.
21. Reis CR, van der Sloot AM, Natoni A, Szegezdi E, Setroikromo R, Meijer M et al. Rapid and efficient cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL

variants. Cell Death Dis 2010; 1:e83.
22. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. Proc Natl Acad Sci U S A 2006; 103(23):8634-8639.
23. Voortman J, Resende TP, bou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 2007; 6(7):2103-2112.
24. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39(7-8):1462-1475.
25. Johnson FM, Saigal B, Talpaz M, Donato NJ. Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. Clin Cancer Res 2005; 11(19 Pt 1):6924-6932.
26. Purnell PR, Mack PC, Tepper CG, Evans CP, Green TP, Gumerlock PH et al. The Src inhibitor AZD0530 blocks invasion and may act as a radiosensitizer in lung cancer cells. J Thorac Oncol 2009; 4(4):448-454.
27. Spierings DC, de Vries EG, Timens W, Groen HJ, Boezen HM, de Jong S. Expression of TRAIL and TRAIL death receptors in stage III non-small cell lung cancer tumors. Clin Cancer Res 2003; 9(9):3397-3405.
28. Gallick GE. SRC as a potential therapeutic target in solid tumor oncology. Clin Adv Hematol Oncol 2004; 2(7):435-437.
29. Mazurenko NN, Kogan EA, Zborovskaya IB, Kisseljov FL. Expression of pp60c-src in human small cell and non-small cell lung carcinomas. Eur J Cancer 1992; 28(2-3):372-377.
30. Guarino M. Src signaling in cancer invasion. J Cell Physiol 2010; 223(1):14-26.
31. Mukhopadhyay UK, Mooney P, Jia L, Eves R, Raptis L, Mak AS. Doubles game: Src-Stat3 versus p53-PTEN in cellular migration and invasion. Mol Cell Biol 2010; 30(21):4980-4995.
32. Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonuccelli G et al. Stat3 promotes metastatic progression of prostate cancer. Am J Pathol 2008; 172(6):1717-1728.
33. Teng TS, Lin B, Manser E, Ng DC, Cao X. Stat3 promotes directional cell migration by regulating Rac1 activity via its activator betaPIX. J Cell Sci 2009; 122(Pt 22):4150-4159.
34. Snyder M, Huang XY, Zhang JJ. Signal transducers and activators of transcription 3 (STAT3) directly regulates cytokine-induced fascin expression and is required for breast cancer cell migration. J Biol Chem 2011; 286(45):38886-38893.
35. Boyer B, Bourgeois Y, Poupon MF. Src kinase contributes to the metastatic spread of carcinoma cells. Oncogene 2002; 21(15):2347-2356.
36. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119(6):1420-1428.
37. van Nimwegen MJ, van de WB. Focal adhesion kinase: a potential target in cancer therapy. Biochem Pharmacol 2007; 73(5):597-609.
38. Cursi S, Rufini A, Stagni V, Condo I, Matafora V, Bachi A et al. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. EMBO J 2006; 25(9):1895-1905.
39. Ishimura N, Isomoto H, Bronk SF, Gores GJ. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 2006; 290(1):G129-G136.
40. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM et al. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and non-apoptotic signals in non small cell lung carcinoma cells. Cancer Res 2007; 67(14):6946-6955.
41. Song JJ, Kim JH, Sun BK, Alcala MA, Jr., Bartlett DL, Lee YJ. c-Cbl acts as a mediator of Src-induced activation of the PI3K-Akt signal transduction pathway during TRAIL treatment.

Cell Signal 2010; 22(3):377-385.
42. Van Schaeybroeck S, Kelly DM, Kyula J, Stokesberry S, Fennell DA, Johnston PG et al. Src and ADAM-17-mediated shedding of transforming growth factor-alpha is a mechanism of acute resistance to TRAIL. Cancer Res 2008; 68(20):8312-8321.
43. Elrod HA, Lin YD, Yue P, Wang X, Lonial S, Khuri FR et al. The alkylphospholipid perifosine induces apoptosis of human lung cancer cells requiring inhibition of Akt and activation of the extrinsic apoptotic pathway. Mol Cancer Ther 2007; 6(7):2029-2038.
44. Kandasamy K, Srivastava RK. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small cell lung cancer cells. Cancer Res 2002; 62(17):4929-4937.

Chapter 5

Inhibition of Hsp90 by 17-AAG enhances TRAIL-induced apoptosis in non-small cell lung cancer cells.

Kaamar Azijli, Saravanan Yuvaraj, Sjors Kas, Godefridus J. Peters, Steven de Jong, Frank A.E. Kruyt

Submitted for publication

ABSTRACT

TRAIL is an interesting cancer therapeutic agent, because of its capability of inducing apoptosis selectively in tumor cells. Unfortunately, half of the NSCLC cells are resistant to TRAIL-induced apoptosis. The rational combination of TRAIL with other agents is known to provide an effective strategy to increase the cell killing potential of TRAIL. Here, we show that targeting Hsp90, an important heat shock protein in the tumorigenesis of NSCLC, with 17-AAG increased TRAIL-induced apoptosis in TRAIL sensitive H460 and resistant A549 cells. The combination index (CI), determined from MTT growth inhibition assays for combined treatment with TRAIL and 17-AAG was 0.28 and 0.44 in A549 and H460 cells, respectively, indicative of strong synergistic activity. 17-AAG increased the level of apoptosis by enhancing activation of the extrinsic/caspase-8 apoptosis pathway. In H460 cells intrinsic-mitochondrial dependent apoptosis was also slightly enhanced. Combined treatment resulted in cleavage of RIP1 and down-regulation of Akt and ERK. Inhibition of Akt activity by LY294002 resulted in a significant increase in TRAIL-induced apoptosis. In conclusion, we found that 17-AAG enhances TRAIL-induced apoptosis in NSCLC by stimulating the extrinsic apoptotic pathway, which could be linked to the suppression of Akt signaling. The combined use of Hsp90 targeting agents and induction of apoptosis by TRAIL receptor agonists might be of therapeutic value in the treatment of NSCLC.

Key Words: TRAIL, 17-AAG, HSP90, NSCLC, synergy, apoptosis

INTRODUCTION

Lung cancer is the most deadliest cancer type in both men and women. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases. Even though, a lot of progress has been made in the treatment of NSCLC in recent years, the 5-years overall survival is still less than 15% [1]. Novel therapies for this type of cancer are therefore urgently needed.

The TNF-related apoptosis inducing ligand (TRAIL) apoptotic pathway is a promising avenue to eradicate tumor cells as it induces cell death specifically in tumor cells and not in normal cells [2;3]. TRAIL can bind as a homotrimer to five different TRAIL- or death receptors (DR), named TRAIL-R1 (DR4) and TRAIL-R2 (DR4), the decoy receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) and the soluble receptor, osteoprotegerin (OPG). When TRAIL binds to TRAIL-R1 or TRAIL-R2, a death inducing signaling complex (DISC) is formed activating the extrinsic pathway, which then results in apoptosis. The decoy receptors and OPG do not transduce death signals into cells, but instead are thought to reduce apoptosis induction via the functional receptors TRAIL-R1 and –R2 by sequestering TRAIL [4]. The extrinsic apoptosis pathway can also crosstalk with the intrinsic apoptosis pathway. This occurs through Bid, which is cleaved by caspase-8, producing truncated (t)Bid that results in activation of the pro-apoptotic Bcl-2 family members, BAX or BAK. These proteins cause induction of mitochondrial outer membrane permeabilization (MOMP) leading to cytosolic release of apoptogenic factors such as cytochrome c, which is a cofactor for caspase-9 activation leading to effector caspases activation [4].

Currently, TRAIL is tested in several clinical trials in which different agonistic agents are used in addition to recombinant produced TRAIL, such as mapatumumab (anti-TRAIL-R1 antibody), AMG 655 (anti-TRAIL-R2 antibody) and CS-1008 [5-7] However, from preclinical studies it is known that around half of the tumor cell lines are refractory to TRAIL-induced apoptosis that can be converted into a sensitive phenotype by combined use with other agents [8]. For example, various chemotherapeutics as well as novel targeted agents can enhance TRAIL-induced apoptosis in NSCLC (see [8] and references therein). Further research is ongoing to identify other or additional mechanisms that affect TRAIL sensitivity and that may be exploited for therapeutic purposes.

TRAIL apoptotic signaling can be suppressed as a result of activity of proteins such as Akt, EGFR, and NF-κB that can modulate this apoptotic pathway in multiple ways [9-12]. The heat shock proteins (Hsp's) are a group of chaperone proteins that are crucial for the maintenance of the stability and function of their client proteins, allowing cells to survive in lethal conditions. Especially Hsp90, is upregulated in many tumors and has an essential role in maintaining the malignant transformation of cancer cells [13]. A role for Hsp90 has also been suggested in the tumorigenesis of NSCLC [14]. Hsp90 interacts with and stabilizes several key signaling proteins, such as Akt, ErbB2, c-Met, and Raf-1. Inhibition of Hsp90 prevents association with client proteins leading to ubiquitination of the unfolded

targets and subsequent proteasomal degradation thus removing possible anti-apoptotic proteins resulting in sensitization for apoptosis [15]. The small molecule, 17-allylamino-17-demethoxy-geldanamycin (17-AAG) is a potent Hsp90 inhibitor that induces cell cycle arrest and apoptosis in a variety of tumor cells, including NSCLC [16].
In this study we investigated whether 17-AAG enhances the apoptosis-inducing effect of TRAIL in NSCLC cells. Furthermore, the mode of action of the synergy of these two compounds was studied.

MATERIALS & METHODS

Cell lines and reagents

The NSCLC cell lines, A549 and H460, were cultured in RPMI-1640 supplemented with 10% Fetal Calf Serum (FCS). The cells were maintained in a humidified incubator at 37 °C and in a 5% CO_2-atmosphere. TRAIL (PeproTech EC Ltd, London, UK) was aliquoted in PBS at a final concentration of 500 ng/ml and stored at -20 °C. 17-AAG was diluted in DMSO (stock solution of 1mM) and stored at -20 °C. The caspase inhibitors Z-VAD-fmk (general caspase inhibitor), Z-IETD-fmk (caspase-8) and Z-LEHD-fmk (caspase-9) were purchased from Bachem AG, dissolved in DMSO (10 mM or 20 mM) and stored at -20 °C.

Drug cytotoxicity assays

Drug cytotoxicity was determined using the methyl thiazole tetrazolium (MTT) assay. Cells were seeded in 96-wells plates with a cell density of 9,000 cells/well. After 24 h, enabling attachment, cells were exposed (in triplicate) to increasing concentrations of the two drugs for 24 h. Next, the medium was removed and 50 µl MTT (10% diluted in HBSS buffer) per well was added and incubated for 1-3 h at 37 °C. After the incubation, 150 µl DMSO per well was added and following 10-15 min slowly shaking, the absorbance of the plate was measured at 540 nm with a TECAN plate reader by using the XFLUOR4 program. IC_{50} values were determined from the graphs. Synergy of TRAIL and 17-AAG was determined by calculation of the combination index (CI) using Calcusyn (Calcusyn Biosoft, Cambridge, UK) as described earlier [17]. For calculation of the CI, only values above a fraction affected (FA) of 0.5 were used, equivalent to 50 - 100% growth inhibition. A CI < 0.9 indicates synergism and >1.1 antagonism.

Cell cycle and cell death measurements

Cells were plated at a density of 200,000 cells/well in 6-wells plates. After 24 h enabling attachment, the medium was replaced by medium containing the drug(s) as indicated. When indicated, caspase inhibitors were added to a final concentration of 20 µM, 2 h prior to drug treatment. After drug exposure cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1200 rpm. After

propidium iodide (PI) staining cell cycle distribution and death cells in the sub-G1 fraction was determined by flow cytometry. Apoptotic events were measured by annexin V-FITC and PI double staining according to the manufacturer's protocol (the Annexin V Apoptosis Detection Kit 1; BD Pharmingen™). Cells stained negative for both annexin V-FITC and PI were living cells. Annexin V-FITC positive cells were defined as early apoptotic cells, and cells positive for both annexin V and PI were defined as late apoptosis. Cells that were only PI positive are necrotic or already dead cells. All analyses were performed on a FACScalibur flowcytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events using CELLQuest software package.

Western Blotting

Cells were seeded in 25-cm^2 flasks with $1*10^6$ cells in 5 ml medium. After 24 h enabling attachment, the medium was refreshed and after 48 h, the desired concentration of the drug(s), TRAIL, 17-AAG or the combination were added for different time-points. Next, the medium was collected and centrifuged at 1,500 rpm, for 5 min at 4°C to pellet floating cells and washed twice with ice-cold PBS. The attached cells were washed twice with ice-cold PBS and were incubated together with the pelleted floating cells for 15 min with lysis buffer from Cell Signalling Technology Inc. (Danvers, MA, USA) supplemented with fresh 0.04% protease inhibitor cocktail (PIC) and 1 mM Na_2VO_3 on ice. After 30 min incubation, the cells were scraped and subjected to centrifugation at 14.000 rpm for 10 min at 4°C. The supernatant was transferred to new vials and protein concentrations were determined by BIO-Rad. 30-60 µg protein was electrophorized on 10 or 12% SDS-PAGE. After transfer of the proteins on polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon™ –FL PVDF, 0.45 µm blocking was performed in Rockland blocking buffer for 1 h at room temperature (RT). Subsequently, membranes were incubated with the following primary antibodies: anti-Akt (#9272), anti-p-Akt (#9271), anti-ERK (#9102), anti-p-ERK (#9101), anti-IκBα (#4812), anti-p-IκBα (#9246), anti-RIP (#3493), anti-caspase-3 (#9662), anti-caspase-8 (#9746), anti-caspase-9 (#9502), anti-PARP (#9542) all from Cell Signalling Technology Inc. Cathepsin B (#IM27L) was purchased from Calbiochem and β-actin (1: 10 000) was from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies were dissolved (1:1000) in PBS-Tween (0.05%) (PBS-T): Rockland buffer (1:1) and the membranes were incubated for 24 h at 4°C. After washing with PBS-T (0.05%), the membranes were incubated with the secondary antibodies (Goat-anti-Rabbit IRDye 800 CW and Goat-anti-Mouse IRDye 680, from LI-COR Biosciences, Lincoln, Nebraska USA) also dissolved in PBS-T: Rockland buffer (1:1) for 1 h at RT. After washing with PBS-T and PBS alone to reduce the background, the fluorescence intensity was measured with the Odyssey Infrared Imaging System (LI-COR Bioscience) and the measurements were evaluated with the program Odyssey V3.0.

RESULTS

Synergistic activity of 17-AAG and TRAIL in NSCLC cells

H460 and A549 cells were treated for 24 h with increasing concentrations of 17-AAG. Both cell lines showed similar sensitivities for this compound (Fig. 1A). The IC_{50} values of 17-AAG were not reached after 24 h incubation. Next, the effect of 17-AAG on TRAIL sensitivity was determined in TRAIL sensitive H460 cells and resistant A549 cells by using MTT assays. TRAIL treatment alone for 24 h effectively killed H460 cells with an IC_{50} value of 5.7 ng/ml, whereas A549 cells were resistant for TRAIL having an IC_{50} of more than 500 ng/ml (Fig. 1). Next, different concentrations of TRAIL and a fixed concentration of 100 nM 17-AAG, representing the IC_{20}, (Fig. 1A) were applied to the cells and the percentage of growth inhibition was determined. The simultaneous administration of 17-AAG and TRAIL showed synergistic activity in A549 and H460 cells, with a CI of 0.28 ± 16 and 0.44 ± 0.23, respectively (Fig. 1B and C).

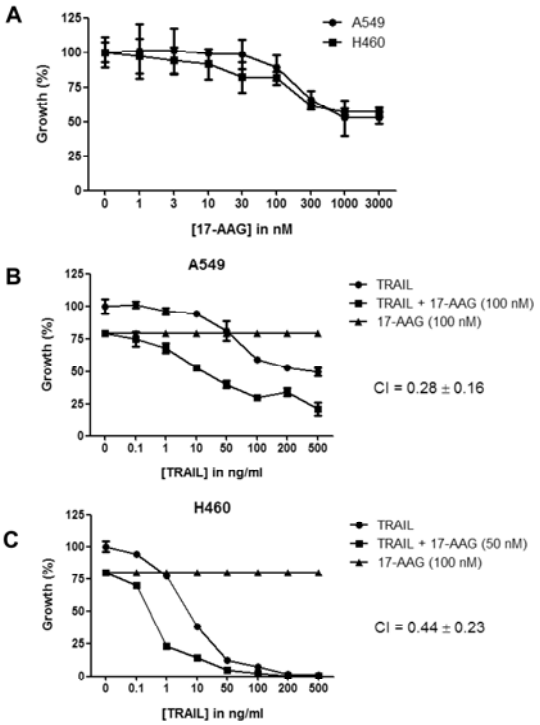

Figure 1. The Hsp inhibitor 17-AAG synergistically enhances cytotoxicity of TRAIL. (**A**) Dose-response curve of 17-AAG in A549 and H460 cells. Cell growth was measured with the MTT assay. Growth inhibition curves of A549 (**B**) and H460 (**C**) cells after 24 h exposure to increasing concentrations of TRAIL alone or in combination with 100 nM 17-AAG. The average CI values were calculated from fraction affected (FA) data points above 0.5. CI's below 0.9 indicate synergy, CIs between 0.9 and 1 indicates additive effects and higher CI values represent antagonism. The CI values ±SEM are averages of three independent experiments.

17-AAG induces cell cycle arrests and enhances TRAIL-induced cell death

In order to examine the mechanism underlying TRAIL and 17-AAG cytotoxicity cell cycle progression was studied of treated cells by flowcytometric analysis of PI stained cells. TRAIL exposure alone in H460 and A549 cells did not alter cell cycle progression (Fig. 2). After 17-AAG treatment however, an increase of around 15% in the portion of A549 cells in the G2/M phase was observed compared to untreated cells; this coincided with a decrease in the proportion of S phase cells (Fig. 2A). In H460 cells no such G2/M arrest by 17-AAG was seen, but rather an approximately 10% increase of cells in the G1 phase was detected together with a similar decrease in cells in S phase (Fig. 2B).

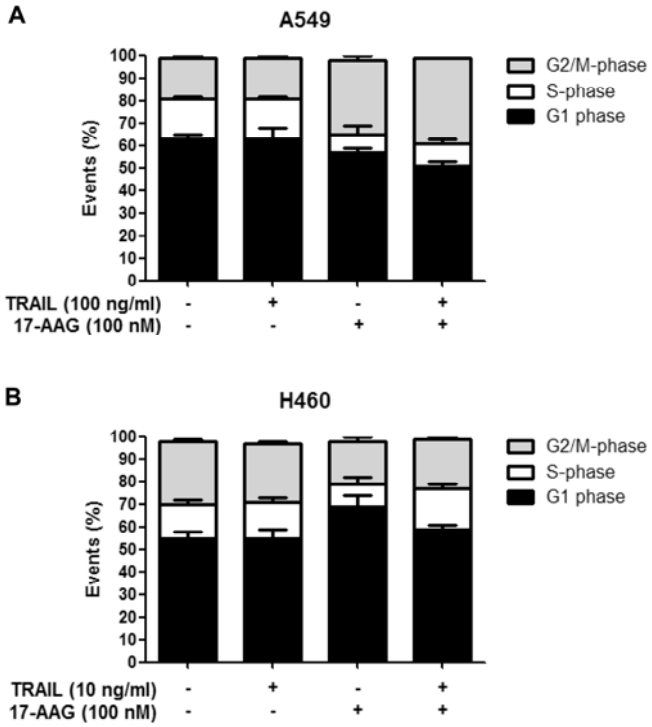

Figure 2. Effect of 17-AAG and TRAIL on cell cycle progression. (**A**) A549 cells were incubated with 100 ng/ml TRAIL, 100 nM 17-AAG or the combination for 24 h. (**B**) The H460 cell line was treated for 24 h with 10 ng/ml TRAIL, 100 nM 17-AAG or the combination of these two drugs. Values are means of at least 2 independent experiments ±s.d.

Cell death was also measured by determining the sub-G1 fraction. A549 cells were treated with TRAIL (50 ng/ml and 100 ng/ml) for 24 h showing only at around 5% of cell death. Simultaneously treatment with 100 nM 17-AAG resulted in an approximately 3-fold increase in sub-G1 cells, whereas 17-AAG alone hardly affected cell death (Fig. 3A). Synergistic cell killing by TRAIL and 17-AAG was also observed in H460 cells, where the percentage of cells in the sub-G1 increased around 3-fold at a low concentration of TRAIL (10 ng/ml) and showed an approximately 1.7-fold increase upon combination with 50

ng/ml TRAIL (Fig. 3B). Apoptotic cell death was more precisely analyzed by determining the level of phosphatidyl serine (PS) exposed on the cell surface following incubation with annexin V-FITC and together with determining PI uptake. 17-AAG combined with TRAIL (2 concentrations) was able to enhance the level of annexin V-FITC-only positive cells by around 3-fold, indicative of early apoptosis, when compared to TRAIL alone treatment (Fig. 3C). In H460 cells 17-AAG addition resulted in an approximately more than 2-fold or 1.5 fold increase in early apoptotic cells treated with a low and high concentration TRAIL, respectively, and when compared to TRAIL alone (Fig. 3D). These apoptotic levels were comparable to the fraction of cells detected in the sub-G1 fraction (Fig. 3A and B). Furthermore, under these conditions no significant differences between TRAIL and the TRAIL/17-AAG combination were detected in late apoptosis (white bars). Cells stained with only PI that define necrotic cell death (grey bars) were not detected. Thus, 17-AAG can enhance apoptosis induced by TRAIL in these NSCLC cells.

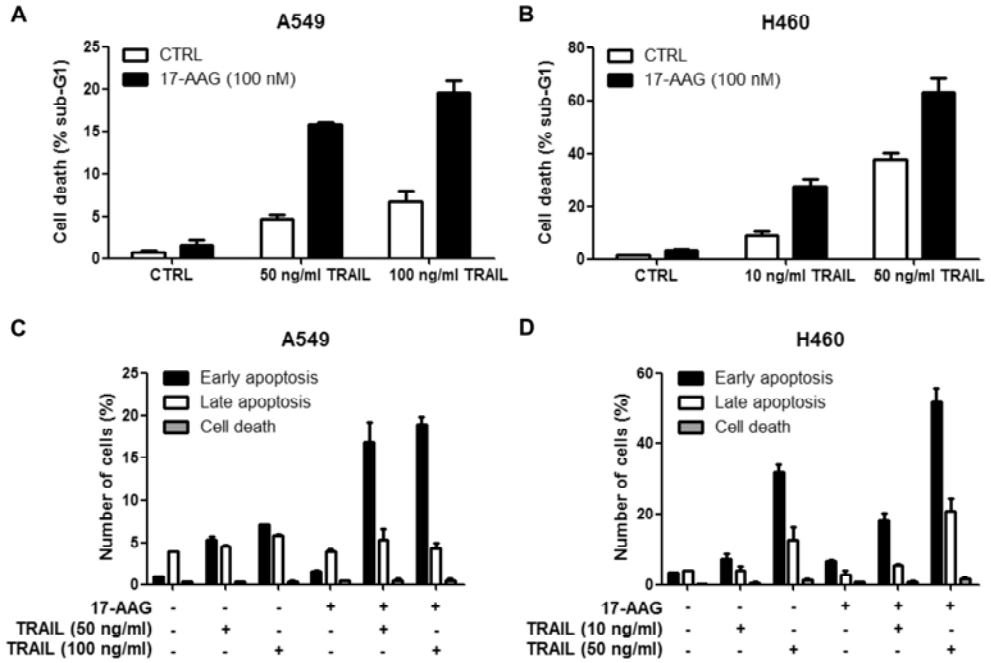

Figure 3. 17-AAG enhances TRAIL-induced apoptosis. (**A**) A549 cells were treated with 50 ng/ml or 100 ng/ml TRAIL with or without 100 nM 17-AAG and the percentage of sub-G1 cells was measured in PI-stained cells following flow-cytometry. (**B**) Sub-G1 levels in H460 cells after 24 h incubation with 10 ng/ml TRAIL, 50 ng/ml TRAIL, 17-AAG (100 nM) or the combination. (**C**) A549 cells treated with TRAIL (50 ng/ml and 100 ng/ml), 17-AAG (100 nM) or the combination for 24 h were double-stained with annexin-V/PI and analyzed by flow cytometry. Distinction can be made between early apoptosis, late apoptosis and cell death. (**D**) Similar experiment with H460 cells exposed to TRAIL (10 ng/ml and 50 ng/ml), 17-AAG (100 nM), or the combination. Values are means of at least two independent experiments ±s.d.

17-AAG enhancement of TRAIL-induced apoptosis is caspases-dependent

To study the role of caspases in the synergistic interaction of TRAIL and 17-AAG, the expression of caspases-8, -9, and -3 were visualized by Western blotting. Time-course experiments showed a slight increase in caspase-8 cleavage after combined treatment compared to TRAIL alone in both A549 and H460 cells after 3 h incubation (Fig. 4A and B). An increase in caspase-9 cleavage after 17-AAG and TRAIL treatment was found in H460 cells from 3 h post-treatment on, as indicated by enhanced detection of cleaved caspase-9 or reduction of the procaspase form, which was not observed in A549 cells. Enhancement of caspase-3 and PARP cleavage by 17-AAG could not be clearly detected in both cell lines.

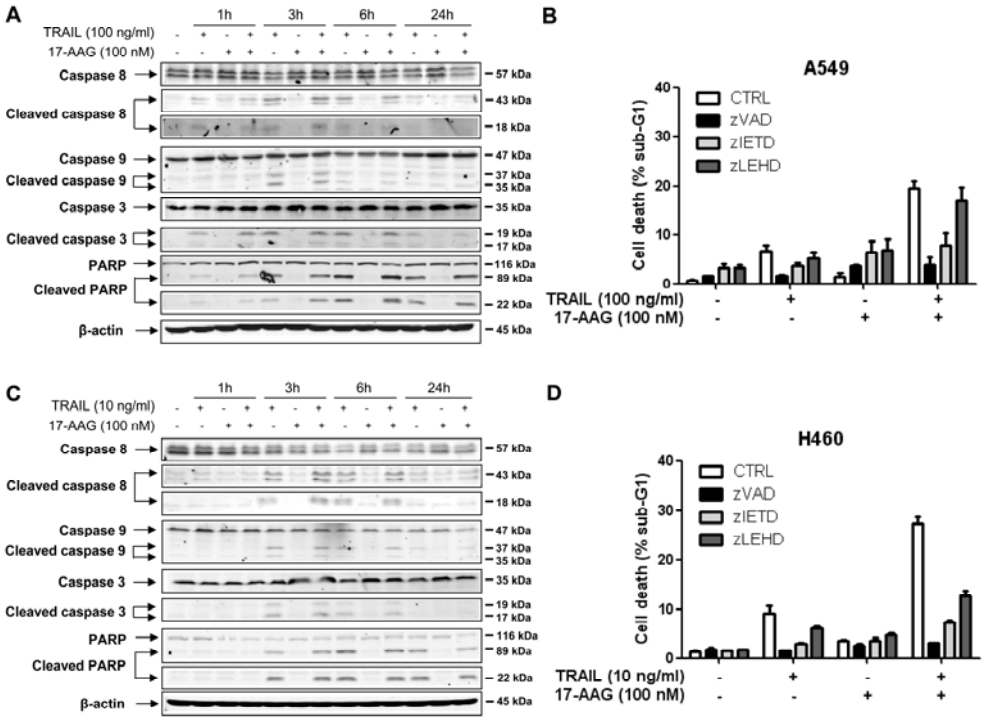

Figure 4. 17-AAG sensitizes for TRAIL in a caspase-dependent manner. (**A**) and (**C**) Representative of two independent Western blots of caspase-8,-9,-3 and PARP of A549 cells after treatment with 100 ng/ml TRAIL, 100 nM 17-AAG or the combination for the indicated time-points. In a similar way, H460 cells were examined after exposure to 10 ng/ml TRAIL or/and 100 nM 17-AAG. As control for protein loading, membranes were probed with an antibody recognizing β-actin. (**B**) and (**D**) Sub-G1 fractions of A549 cells pre-treated with the pancaspase inhibitor zVAD-fmk (20 μM), zIETD-fmk (20 μM), or zLEHD-fmk (20 μM) for 2 h and subsequent treatment with either TRAIL (100 ng/ml), 17-AAG (100 nM) or the combination of both drugs for 24 h. Similarly, H460 cells were treated with TRAIL (10 ng/ml), 17-AAG (100 nM) or the combination for 24 h. Means of triplicate determinations ±SEM.

The involvement of caspases in the sensitizing effect of 17-AAG on TRAIL-induced apoptosis was further examined by using synthetic caspases inhibitors. The broad-caspase inhibitor z-VAD-FMK (20 μM) completely inhibited TRAIL-induced apoptosis determined

by sub-G1 levels in the absence and presence of 17-AAG in A549 and H460 cells (Fig. 4C and D). Inhibition of caspase-8 by the synthetic inhibitor z-IETD-FMK (20 μM) also prevented synergistic apoptosis activation by TRAIL and 17-AAG in both cell lines. However, the caspase-9 inhibitor, z-LEHD-FMK (20 μM) decreased sub-G1 levels after TRAIL/ 17-AAG exposure only in H460 cells and not in the A549 cell line. Overall, these findings are in line with the caspase cleavage patterns observed in the Western blots.

Effects of 17-AAG and TRAIL on client proteins of Hsp90

Hsp90 stabilizes multiple oncoproteins such as EGFR, Her-2, NF-κB, and Akt. In addition, RIP1 has also been found to be a target of Hsp90 inhibitors [18;19]. We evaluated the expression and phosphorylation status of a number of client proteins that could be involved in mediating the synergistic effect of 17-AAG and TRAIL. RIP1 is part of the secondary signaling complex known to be involved in pro-survival signaling by TRAIL [20]. The TRAIL/17-AAG combination decreased the expression of RIP1 in A549 cells in particular after 6 h incubation, which was accompanied with an increase in RIP1 cleavage. After 24 h incubation this effect on RIP1 was reduced. H460 cells treated with TRAIL resulted in RIP1 cleavage that was not clearly enhanced by addition of 17-AAG. However, as shown previously silencing of RIP1 expression with a short hairpin RNA did not sensitize the resistant A549 cells for TRAIL-induced apoptosis, making a role of RIP1 in 17-AAG-dependent sensitization unlikely [21]. Activation of ERK, Akt and NF-κB can negatively regulate TRAIL-induced apoptosis and activation of these pathways has been correlated with TRAIL resistance in NSCLC cells as we and others have previously shown [10;11;21]. Therefore, we extended our study to examine the effect of 17-AAG on Akt, ERK and IκB/NF-κB. We observed TRAIL-induced Akt and ERK phosphorylation in the resistant A549 cells and levels were attenuated by 17-AAG to different extents (Fig. 5A). In H460 cells, p-ERK levels were decreased after 17-AAG exposure for 6 and 24 h. TRAIL treatment had no effect on p-ERK levels in these sensitive cells. TRAIL treatment for 1 h induced IκBα phosphorylation only in A549 cells, but co-incubation with 17-AAG did not abrogate the phosphorylation of this kinase.

Next, we examined the expression of survivin in NSCLC cells. Survivin is a member of the Inhibitor of Apoptosis (IAP) protein family and regulates apoptosis by inhibiting effector-caspases. Survivin, which interacts with Hsp90 [22], has been identified to play an important role in the sensitizing effect of 17-AAG in TRAIL-induced apoptosis in malignant gliomas, as 17-AAG downregulated the expression of this protein [23].

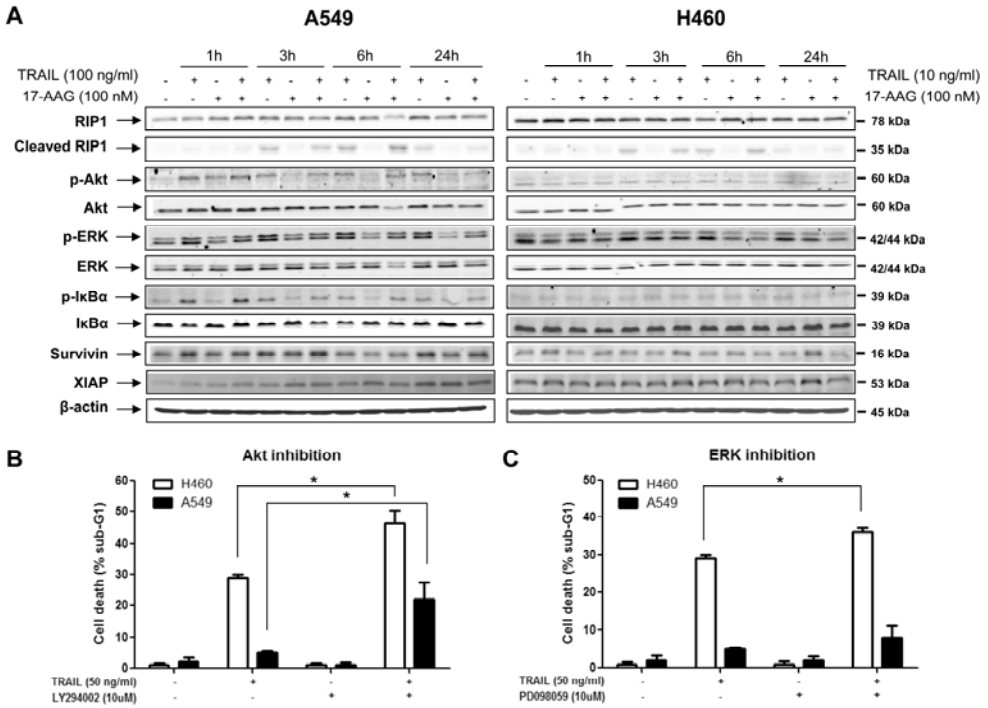

Figure 5. The effect of 17-AAG on Hsp90 client proteins possibly involved in TRAIL sensitization. (**A**) A549 and H460 cells were treated with TRAIL, 17-AAG or the combination with the indicated concentrations for different time-points and the expressions of RIP1, p-Akt/ Akt, p-ERK/ ERK, p-IκBα/ IκBα, Survivin and XIAP were determined by Western blotting. Representative blots of two independent experiments are shown. β-Actin was taken as loading control. (**B**) H460 and A549 cells were treated with 50 ng/ml TRAIL for 24 h with or without the Akt inhibitor, LY294002 (10 μM) or (**C**) the ERK inhibitor, PD098059 (10 μM). Cells were stained with PI, analyzed by flow cytometry, and sub-G1 levels were determined. Each point represents the mean ± SEM of three independent experiments. * $p<0.05$, t test.

In A549 cells, TRAIL increased the expression of survivin. 17-AAG did not suppress survivin protein levels, suggesting that this protein is not likely to play a regulatory role in the synergistic interaction of TRAIL and 17-AAG in this cell line, and neither in the H460 cell line. Expression of XIAP, also a member of the IAP protein family, was reported to decrease in TRAIL/17-AAG treated colon cancer cells [24]. However, no significant changes in XIAP levels were detected in both NSCLC cell lines (Fig. 5A). To further examine the role of Akt and ERK activities in TRAIL sensitivity, cells were co-treated with an Akt inhibitor, LY294002, or an ERK inhibitor, PD098059. LY294002 significantly increased the number of cells with sub-G1 content from 29 ±1% to 46 ±4.2% in H460 cells and from 5 ±0.5% to 22 ±5.5% in A549 cells. However, PD098059 exposure increased TRAIL-induced apoptosis significantly only in H460 cells from 29 ±1% to 36 ±1.15% (Fig. 5B,C).

DISCUSSION

In the present study, we found synergistic activity between 17-AAG and TRAIL in TRAIL-sensitive H460 and TRAIL-resistant A549 cells. Hsp90 is amongst others a chaperone of proteins regulating cell cycle progression [25]. Cell cycle arrest in the G2-M phase by Hsp90 inhibitors has been related to Plk1 [26]. In Hodgkin's lymphoma cells, 17-AAG decreased the expressions of Plk1 and cyclin B1, which regulates the transition of G2-M phase to mitosis and is activated by Plk1 [27]. Although 17-AAG affected cell cycle progression differently in A549 and H460 cells, inducing a G2/M-arrest versus a G1 arrest, respectively, synergistic activity was the result of enhanced apoptosis activation.

17-AAG enhanced TRAIL-induced cell death in H460 cells, and sensitized resistant A549 cells for TRAIL. The synergistic cytotoxicity was caspase-dependent; 17-AAG stimulated caspase-8 activation in both cell lines. In H460 cells, enhanced caspase-9 activation was also involved in increased apoptosis activation. In order to obtain insight in the underlying mechanism of apoptosis sensitization by 17-AAG we have examined several Hsp90 chaperones that are known to regulate apoptosis.

RIP1 and IκB are two essential components of the NF-κB pathway and are client proteins of Hsp90 [28]. RIP1 expression was reported to decrease upon Hsp90 inhibition leading to sensitization for TRAIL-induced apoptosis in breast and lung cancer cells [18;19]. Inhibition of NF-κB as a molecular mechanism of 17-AAG-dependent TRAIL sensitization has been described in colon and lung cancer cells [18;24]. In the present study, 17-AAG resulted in a decrease in RIP expression in A549 cells that was accompanied by an increase in RIP cleavage. This was not observed in H460 cells that already demonstrate RIP1 cleavage following TRAIL treatment. Since RIP1 silencing in A549 cells does not lead to sensitization for TRAIL [21], RIP1 is unlikely to be the primary mediator of the effect of 17-AAG on TRAIL sensitivity in our study. TRAIL-dependent phosphorylation of IκBα, leading to degradation of this inhibitory protein and activation of the NF-κB pathway was observed only in TRAIL resistant A549 cell line. Several studies have shown that the activation of NF-κB confers resistance to TRAIL-induced apoptosis, including NSCLC, and inhibition of NF-κB can lead to TRAIL-sensitization [11;29;30]. However, 17-AAG did not attenuate the phosphorylation of IκBα induced by TRAIL, making this pathway an unlikely effector of 17-AAG activity in our study.

IAP family members, in particularly XIAP and survivin have also been linked to resistance to TRAIL-induced apoptosis [31]. In resistant glioma cells, 17-AAG sensitized for TRAIL by reducing survivin levels through enhanced proteasomal degradation [23]. Furthermore, the 17-AAG/TRAIL combination has been reported to cause down-regulation of XIAP, leading to enhanced caspase-3 activation in colon cancer cells [24]. In the present study, we found no differences in survivin and XIAP expression in NSCLC cell lines between single and combined treatment with 17-AAG and TRAIL.

Examination of Akt revealed an inhibitory effect of 17-AAG on Akt phosphorylation only in

A549 cells. In H460 cells, minimal TRAIL-induced Akt phosphorylation is detectable despite the observation that the PI3K/Akt inhibitor LY294002 to some extent increased TRAIL-induced apoptosis. In resistant A549 cells, LY294002 co-treatment enhanced TRAIL-induced apoptosis by 4-fold. Thus, Akt could be a mediator of the 17-AAG effect in NSCLC cells. Perifosine, an Akt inhibitor, was described to increase TRAIL-R2 receptor expression and decrease the cellular FLICE-inhibitory protein (cFLIP) in human lung cancer cell lines, enhancing caspase-8 activation [32]. ERK has generally been linked to TRAIL-induced proliferation [33;34]. The inhibition of ERK by PD098059 did hardly sensitize these NSCLC cells for TRAIL and thus is unlikely to contribute to 17-AAG enhancement of apoptosis.
Clinical trials using small molecule inhibitors of Hsp90, like 17-AAG, 17-DMAG, VER-52269, CNF2024, and IPI-504 are currently being conducted [35]. Based on our results and previous studies, targeting Hsp90 in combination with induction of apoptosis by TRAIL might be of therapeutic value in the treatment of NSCLC.

Acknowledgements

This research was performed within the framework of project T3-112 and T3-103 of the Dutch Top Institute Pharma.

Reference List

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60(5):277-300.
2. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev 2003; 14(3-4):337-348.
3. Kruyt FA. TRAIL and cancer therapy. Cancer Lett 2008; 263(1):14-25.
4. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
5. Forero-Torres A, Shah J, Wood T, Posey J, Carlisle R, Copigneaux C et al. Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). Cancer Biother Radiopharm 2010; 25(1):13-19.
6. Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W et al. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008; 61(1):82-90.
7. Herbst RS, Kurzrock R, Hong DS, Valdivieso M, Hsu CP, Goyal L et al. A first-in-human study of conatumumab in adult patients with advanced solid tumors. Clin Cancer Res 2010; 16(23):5883-5891.
8. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
9. Eid MA, Lewis RW, Abdel-Mageed AB, Kumar MV. Reduced response of prostate cancer cells to TRAIL is modulated by NFkappaB-mediated inhibition of caspases and Bid activation. Int J Oncol 2002; 21(1):111-117.
10. Kandasamy K, Srivastava RK. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small cell lung cancer cells. Cancer Res 2002; 62(17):4929-4937.
11. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM et al. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and non-apoptotic signals in non small cell lung carcinoma cells. Cancer Res 2007; 67(14):6946-6955.
12. Van Schaeybroeck S, Kelly DM, Kyula J, Stokesberry S, Fennell DA, Johnston PG et al. Src and ADAM-17-mediated shedding of transforming growth factor-alpha is a mechanism of acute resistance to TRAIL. Cancer Res 2008; 68(20):8312-8321.
13. Altieri DC. Coupling apoptosis resistance to the cellular stress response: the IAP-Hsp90 connection in cancer. Cell Cycle 2004; 3(3):255-256.
14. Gallegos Ruiz MI, Floor K, Roepman P, Rodriguez JA, Meijer GA, Mooi WJ et al. Integration of gene dosage and gene expression in non-small cell lung cancer, identification of HSP90 as potential target. PLoS One 2008; 3(3):e0001722.
15. Zuehlke A, Johnson JL. Hsp90 and co-chaperones twist the functions of diverse client proteins. Biopolymers 2010; 93(3):211-217.
16. Nguyen DM, Chen A, Mixon A, Schrump DS. Sequence-dependent enhancement of paclitaxel toxicity in non-small cell lung cancer by 17-allylamino 17-demethoxygeldanamycin. J Thorac Cardiovasc Surg 1999; 118(5):908-915.
17. Bijnsdorp IV, Kruyt FA, Gokoel S, Fukushima M, Peters GJ. Synergistic interaction between trifluorothymidine and docetaxel is sequence dependent. Cancer Sci 2008; 99(11):2302-2308.
18. Wang X, Ju W, Renouard J, Aden J, Belinsky SA, Lin Y. 17-allylamino-17-demethoxygeldanamycin synergistically potentiates tumor necrosis factor-induced lung cancer cell death by blocking the nuclear factor-kappaB pathway. Cancer Res 2006; 66(2):1089-1095.

19. Palacios C, Lopez-Perez AI, Lopez-Rivas A. Down-regulation of RIP expression by 17-dimethylaminoethylamino-17-demethoxygeldanamycin promotes TRAIL-induced apoptosis in breast tumor cells. Cancer Lett 2010; 287(2):207-215.
20. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
21. Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. J Cell Sci 2012.
22. Fortugno P, Beltrami E, Plescia J, Fontana J, Pradhan D, Marchisio PC et al. Regulation of survivin function by Hsp90. Proc Natl Acad Sci U S A 2003; 100(24):13791-13796.
23. Siegelin MD, Habel A, Gaiser T. 17-AAG sensitized malignant glioma cells to death-receptor mediated apoptosis. Neurobiol Dis 2009; 33(2):243-249.
24. Vasilevskaya IA, O'Dwyer PJ. 17-Allylamino-17-demethoxygeldanamycin overcomes TRAIL resistance in colon cancer cell lines. Biochem Pharmacol 2005; 70(4):580-589.
25. Burrows F, Zhang H, Kamal A. Hsp90 activation and cell cycle regulation. Cell Cycle 2004; 3(12):1530-1536.
26. de CG. Heat shock protein 90 regulates the metaphase-anaphase transition in a polo-like kinase-dependent manner. Cancer Res 2004; 64(15):5106-5112.
27. Georgakis GV, Li Y, Rassidakis GZ, Martinez-Valdez H, Medeiros LJ, Younes A. Inhibition of heat shock protein 90 function by 17-allylamino-17-demethoxy-geldanamycin in Hodgkin's lymphoma cells down-regulates Akt kinase, dephosphorylates extracellular signal-regulated kinase, and induces cell cycle arrest and cell death. Clin Cancer Res 2006; 12(2):584-590.
28. Arya R, Mallik M, Lakhotia SC. Heat shock genes - integrating cell survival and death. J Biosci 2007; 32(3):595-610.
29. Eid MA, Lewis RW, Abdel-Mageed AB, Kumar MV. Reduced response of prostate cancer cells to TRAIL is modulated by NFkappaB-mediated inhibition of caspases and Bid activation. Int J Oncol 2002; 21(1):111-117.
30. Jeremias I, Kupatt C, Baumann B, Herr I, Wirth T, Debatin KM. Inhibition of nuclear factor kappaB activation attenuates apoptosis resistance in lymphoid cells. Blood 1998; 91(12):4624-4631.
31. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005; 12(3):228-237.
32. Elrod HA, Lin YD, Yue P, Wang X, Lonial S, Khuri FR et al. The alkylphospholipid perifosine induces apoptosis of human lung cancer cells requiring inhibition of Akt and activation of the extrinsic apoptotic pathway. Mol Cancer Ther 2007; 6(7):2029-2038.
33. Belyanskaya LL, Ziogas A, Hopkins-Donaldson S, Kurtz S, Simon HU, Stahel R et al. TRAIL-induced survival and proliferation of SCLC cells is mediated by ERK and dependent on TRAIL-R2/DR5 expression in the absence of caspase-8. Lung Cancer 2008; 60(3):355-365.
34. Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D et al. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. Circulation 2003; 107(17):2250-2256.
35. Jhaveri K, Taldone T, Modi S, Chiosis G. Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. Biochim Biophys Acta 2012; 1823(3):742-755.

Chapter 6

The novel thymidylate synthase inhibitor Trifluorothymidine (TFT) and TRAIL synergistically eradicate non-small cell lung cancer cells

Kaamar Azijli, Ingrid van Roosmalen, Jorn Smit, Saravanan Yuvaraj, Masakazu Fukushima, Steven de Jong, Godefridus J. Peters, Irene V. Bijnsdorp, Frank A.E. Kruyt

Submitted for publication

ABSTRACT

TRAIL is a tumor selective anti-cancer agent that may be used for the treatment of non-small cell lung cancer (NSCLC). However, TRAIL resistance is frequently encountered. Here, the combined use of TRAIL with TFT, a thymidylate synthase inhibitor that causes DNA damage, was examined for sensitizing NSCLC cells to TRAIL. Interactions between TRAIL and TFT were studied in NSCLC cell culture models using growth inhibition and apoptosis assays. Western blotting and flow cytometry were used to investigate underlying mechanisms of tumor cell eradication. The combined treatment of TFT and TRAIL showed synergistic cytotoxicity in A549, H292, H322 and H460 cells. For synergistic activity the sequence of administration was important; TFT treatment followed by TRAIL exposure did not show sensitization. Combined TFT and TRAIL treatment for 24 h followed by 48 h of TFT alone was synergistic in all cell lines, with combination index (CI) values below 0.9. The treatments affected cell cycle progression, with TRAIL inducing a G1 arrest and TFT a G2/M arrest. TFT activated Chk2 and reduced Cdc25c levels known to cause G2/M arrest. TRAIL-induced caspase-dependent apoptosis was enhanced by TFT, whereas TFT alone mainly induced caspase-independent death. Further analyses showed that TFT enhanced the expression of p53 and p21/WAF1, and p53 was involved in the increase of TRAIL-R2 surface expression. TFT also caused down-regulation of cFLIP and XIAP and increased Bax expression. TFT enhances TRAIL-induced apoptosis in NSCLC cells by sensitizing the apoptotic machinery at different levels in the TRAIL pathway. Our findings suggest a possible therapeutic benefit of the combined use of TFT and TRAIL in NSCLC.

Key Words: TRAIL, TFT, synergy, apoptosis, NSCLC

INTRODUCTION

Lung cancer is the leading cause of cancer-related death world-wide [1]. Non-small cell lung cancers (NSCLC) are epithelial tumors that represent around 80% of all lung carcinomas. In NSCLC almost half of all cases have locally advanced or widespread metastatic disease at diagnosis, with an overall 5-year survival rate of approximately 1 to 5% [2]. Whereas surgery is the mainstay treatment for localized disease, most patients are candidates for systemic or adjuvant chemotherapy. It has become clear in recent years that a therapeutic plateau has been reached for patients with advanced stage NSCLC treated with conventional chemotherapeutic agents. Therefore, novel chemotherapeutics or targeted therapeutics are required to improve prognosis of these patients.

A potential useful group of agents are the tumor necrosis factor apoptosis-inducing ligand (TRAIL) receptor targeting agents that can activate apoptosis directly in tumor cells, while healthy cells are not affected [3]. TRAIL activates the extrinsic apoptotic pathway after binding cell surface membrane-localized TRAIL death receptors (DRs) that include TRAIL-R1 (DR4), TRAIL-R2 (DR5) and two decoy receptors (TRAIL-R3 (Dc1) and TRAIL-R4 (Dc2) that do not possess functional death domains [4]. After TRAIL binding to TRAIL-R1 or TRAIL-R2 several proteins are recruited to the intracellular receptor death domain, forming the death- inducing signaling complex (DISC) in which caspase-8 is activated. Caspase-8 on its turn can activate downstream caspases, such as caspase-3, or cross-activate mitochondrial apoptosis via the cleavage of the Bcl-2 family member Bid. The mitochondrial or intrinsic pathway involves mitochondrial outer membrane permeabilization (MOMP), which is regulated by the Bcl-2 family of proteins [5]. Cleaved Bid can induce MOMP, leading to the release of pro-apoptotic factors, such as cytochrome c and Smac/DIABLO into the cytosol. Cytochrome c facilitates the formation of the apoptosome in which procaspase-9 is activated, and Smac/DIABLO can sequester X-linked inhibiter of apoptosis (XIAP) thereby preventing XIAP-mediated binding and inhibition of caspase-9 and -3 [6;7]. At the DISC level, cellular FLICE-inhibitory protein (cFLIP) is a potent inhibitor of procaspase-8 activation. Two variants, $cFLIP_L$ and $cFLIP_S$ representing inactive procaspase-8 analogs, have been found to prevent procaspase-8 activation [8]. Currently, several TRAIL-receptor targeting drugs are evaluated in clinical phase I/II trials alone or in combinations for the treatment of NSCLC [9]. However, approximately 50% of tumor cells are resistant to TRAIL and combination with other agents can sensitize tumor cells for TRAIL [9].

TFT is a thymidylate synthase (TS) inhibitor that interferes with thymidylate production and in its triphosphate form can be incorporated into the DNA causing DNA damage [10]. TFT has been found to induce apoptotic cell death in both colon and lung cancer cell lines. Moreover, apart from activating intrinsic and/or extrinsic apoptotic pathways also caspase-independent modes of cell death can be induced by TFT [11]. The lysosomal protease cathepsin B is known to be involved in caspase-independent cell death upon

various stress stimuli [12]. Previously, we found that cathepsin B plays a role in TFT-mediated cell death in colorectal cancer [11]. TFT is part of the formulation TAS-102, in which TFT is combined with the thymidine phosphorylase inhibitor (TPI). TPI inhibits thymidine phosphorylase, which inactivates TFT, increasing its *in vivo* activity [10]. TAS-102 is active in tumor cells resistant to the anti-metabolite 5-fluorouracil (5-FU) suggesting at least partially non-overlapping mechanisms of action [13]. Currently, it is being evaluated in phase II studies for the treatment of several solid tumors [14] and it has shown activity in 5-FU resistant colon cancer cells [15].
In this study, we examined the interaction between TFT and TRAIL in NSCLC cells as a possible novel combination treatment. The effects on cell cycle progression and cell death activation, and underlying molecular mechanisms were explored.

MATERIALS & METHODS

Cell lines and chemicals

Human NSCLC cell lines A549, H292, H322 and H460 were obtained from the American Type Culture Collection (ATCC, Teddington, UK) and were grown as monolayers in 25 cm^2 culture flasks (Greiner Bio-One, Frickenhansen, Germany) at 37°C in a humidified 5% CO_2 atmosphere. The cells were cultured in RPMI, supplemented with 10% foetal calf serum, and 100 units/ml penicillin and streptomycin (Lonza, Verviers, Belgium). TFT (Taiho Pharmaceuticals Co., Ltd, Tokushima, Japan) was dissolved in PBS as a stock solution of 20 mM and was stored in aliquots at -20°C. Aliquots of TRAIL (Peprotech, Rocky Hill, NJ, USA) were stored at -80°C. The synthetic pan-caspase inhibitor zVAD-fmk was obtained from Bachem AG (Bubendorf, Switzerland), and was dissolved in DMSO (Sigma-Alderich, Steinheim, Germany) at 10 mM stock solutions and stored at -20°C. Anti-caspase-3 (#9662), anti-caspase-8 (#9746), anti-caspase-9 (#9502), anti-cleaved caspase-3 (#9661), anti-Chk1 (#2345), anti-phosphorylated Chk1 (Ser345; #2341), anti-Chk2 (#2662), anti-phosphorylated Chk2 (Thr68; #2661), anti-Cdc25c (#4688) and anti-phosphorylated Cdc25c (Ser216; #4901), anti-p53 (#9282), anti-FLIP (#3210), anti-XIAP (#2042) antibodies were all purchased from Cell Signalling Technology (Danvers, MA, USA) and anti-p21 (#sc-756) was from Santa Cruz (Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA). Anti-cathepsin B antibody was purchased from Oncogene Research Products (Boston, MA, USA), anti-β-actin antibody from Sigma-Aldrich Chemicals, Goat-a-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) and goat-a-rabbit-IRDye (800CW;926-32211 and 680;#926-32221) were obtained from Licor (Westburg, Leusden, The Netherlands).

Growth inhibition assay

Drug cytotoxicity was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [16]. Cells (2000/well) were seeded in 96-well plates (Greiner

Bio-One, Frickenhausen, Germany). After 24 h, enabling attachment, cells were exposed to increasing concentrations of TFT for 72 h or a fixed concentration of TRAIL for 24 h (based on the IC_{50}). Two combination schedules were evaluated (Fig. 1A). First, cells were exposed for 24 h to both TFT and TRAIL, followed by 48 h exposure to TFT alone (Schedule A). Second, cells were exposed for 48 h to TFT, after which the combination of TFT with TRAIL was added for 24 h (Schedule B). After drug exposure (72 h in total), the medium was removed and the cells were incubated for 3 h with 50 µl/well of 1 mg/ml MTT solution in phenol red free DMEM (Lonza) at 37°C. Subsequently, 150 µl DMSO was added to each well and the optical density (OD) was measured at 540 nm (Tecan, Männedorf, Switzerland). Differences between OD of the treated and untreated controls were compared to calculate cell growth. From the growth inhibition curves, a combination index (CI) was calculated using CalcuSyn software from Biosoft (Cambridge, UK), based on the median-drug-effect method as described previously [10]. A CI < 0.9 indicates synergism and > 1.1 antagonism. For calculation of the CI, only values above a fraction affected (FA) of 0.5 were used, equivalent to 50 – 100% growth inhibition. FA values below 0.5 are considered to be irrelevant, because these represent only a minor growth inhibition. Per experiment the CI values at FA higher than 0.5 were averaged and the mean was used for comparison of separate experiments.

Cell cycle and cell death analyses

Cell cycle analysis and cell death measurements were performed by FACS analysis as described previously [17]. Cells were seeded in 6-well plates at a density of 150,000 cells/well. After drug exposure, cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1200 rpm. Subsequently, cells were stained with propidium iodide buffer (0.1 mg/ml with 0.1 % RNAse A) on ice in the dark. DNA content of the cells was analyzed by FACSCalibur flowcytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G_1 peak was used to determine the extent of cell death.

Western blotting

Western blotting was performed as described previously [18]. Cells were exposed to TFT, TRAIL or the combination for 24, 48 or 72 h, after which cells were washed twice with ice-cold PBS and disrupted in lysis buffer (Cell Signalling Technology Inc.) supplemented with 0.04% protease inhibitor cocktail (Roche, Almere, the Netherlands). Cell lysates were scraped, transferred into a vial and centrifuged at 11,000 g at 4°C for 10 min. Protein concentrations were determined by the Bio-Rad assay, according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the Netherlands). From each condition 30-80 µg of protein was separated on an 8-12% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon^{TM} –FL PVDF, 0.45 µm).

Subsequently, the membranes were blocked for 1 h at room temperature (RT) in Odyssey blocking buffer (Odyssey blocking buffer #927-40003, Westburg, Leusden, the Netherlands) and incubated overnight at 4°C with the primary antibodies (dilution 1:1,000-10,000 in Odyssey blocking buffer 1:1 diluted with PBS-T (PBS with 0.05% Tween-20). The membrane was washed 5 times in PBS-T and incubated with the secondary antibody (1:10,000 goat-α-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) or goat-α-rabbit-IRDye (800CW;926-32211 and 680;#926-32221), Westburg) for 1 h at RT in the dark. After incubation, the membrane was washed in PBS-T and once with PBS followed by imaging using an Odyssey Infrared Imager (Westburg), at a 84 µm resolution, 0 mm offset and with high quality [19].

Caspase activity

Effects of treatment on the activity of caspase-3, -8 and -9 were determined by fluorometric assay kits (Zebra Bioscience, Enschede, Netherlands), according to manufacture's instructions. In brief, after drug exposure cell pellets were made in ice-cold PBS containing 1 x 10^6 cells, which were stored at -80°C until analysis. Fluorescence was detected at 350 nm excitation and 460 nm emission (Spectra fluor Tecan, Salzburg, Austria). Relative caspase activity was calculated in ratio compared to the untreated control (set to 1).

TRAIL receptor expression

The levels of TRAIL-R1 and TRAIL-R2 expression on cellular membranes were determined by FACS analysis [20]. One million cells untreated or treated with TFT for 24 h were added to a FACS tube and stained with receptor-specific mAbs (TRAIL-R1 mouse anti human Alexis (Alx-804-297) and TRAIL-R2 (Alx-804-298) for 1 h at 4°C. An IgG1 antibody (DAKO) was used as negative control. After washing, cells were incubated with Goat anti-mouse PE labeled (Alexa-488) for 30 min on ice in the dark. Next, the cells were washed and the fluorescence was measured on a FACSCalibur flowcytometer using CELLQuest software (Becton Dickinson, MountainView, CA).

RNA interference

Silencing p53 was performed as described previously [21]. The following p53 siRNAs were used, 5'GCAUGAACCGGAGGCCCAU-dTdT3' (sense) and 5'AUGGGCCUCCGGUUCAUGC-dTdT3' (anti-sense). The negative control siRNA used was from Invitrogen (Breda, the Netherlands). Cells seeded in 6-wells plates were incubated in unsupplemented Optimem® medium and transfected with 133 nM siRNA using Oligofectamine® reagent according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands). The next day, cells were treated with TFT for 24 h, and used for receptor expression analysis with FACS experiments and western blotting.

RESULTS

TRAIL, TFT and combined treatment

The sensitivities of the NSCLC cell lines to TFT and TRAIL are summarized in Table 1. IC_{50} values between 2.9 µM and 6.3 µM were observed for TFT. H292 and H460 cells were TRAIL-sensitive, H322 cells were moderately sensitive and A549 cells were resistant to TRAIL up to the highest tested concentration of 1500 ng/ml. The IC_{50} values of TRAIL for a particular cell line were used in the combination experiments, except for resistant A549 cells where we used a TRAIL concentration of 150 ng/ml.

Table 1 | Growth inhibition and synergy analyses for different treatment schedules of TFT combined with TRAIL

Cell line	Growth inhibition (IC_{50})		Combination index (CI)	
	TFT (µM)	TRAIL (ng/ml)	24 h Combination + 48 h TFT (schedule A)	48 h TFT + 24 h Combination (schedule B)
A549	6.3 ± 1.5	>1500	0.6 ± 0.2	3.7 ± 1.3
H292	3.3 ± 0.8	25 ± 7	0.6 + 0.2	1.6 ± 0.3
H322	2.9 ± 0.2	80 ± 10	0.7 ± 0.3	1.3 ± 0.4
H460	4.2 ± 0.9	10 ± 2	0.6 ± 0.1	2.8 ± 0.9

Combination index (CI) values were calculated from the fraction affected data points from 0.5 to 0.9. CI values lower than 0.9 specify synergism; CI between 0.9-1.0 indicate an additive effect; CI values greater than 1.1 denote antagonism.

Synergistic effects are illustrated as fraction affected (FA)-combination index (CI) plots obtained in H460 and A549 cells (Fig. 1). The combination schedule of 24 h TFT and TRAIL followed by 48 h of TFT alone (Schedule A) was synergistic in all tested cell lines (CI < 0.9), including resistant A549 cells. In contrast, a schedule of 48 h TFT treatment followed by 24 h TFT and TRAIL (Schedule B) appeared to have antagonistic activity in all cell lines examined (Table 1). As TRAIL exerts its apoptotic function quite rapidly, the cells were incubated for just 24 h. TFT, on the other hand, has a long term effect; an incubation time of 72 h of this compound was therefore chosen. In the experiments described below the synergistic schedule, schedule A (24 h combination + 48 h TFT) was selected to further explore the mechanism underlying synergism of TRAIL and TFT.

A

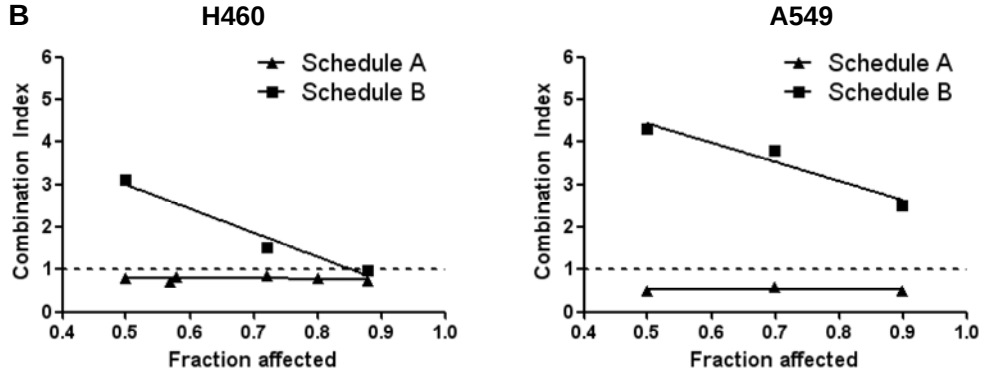

Figure 1. Representative FA-CI plot of combined TFT and TRAIL treatment in H460 and A549 cells using two different exposure schedules as indicated. (**A**) Cells were exposed to increasing concentrations of TFT for 72 h including 24 h exposure to a fixed IC_{50} concentration of TRAIL. TRAIL was added either the first 24 h, or the last 24 h in the presence of TFT and cytotoxicity was determined by MTT assays. (**B**) An average CI was calculated for data-points with FA higher than 0.5.

Effects on cell cycle progression

The effects on cell cycle distribution were analyzed in time-course experiments using flowcytometry on PI-stained cells (Fig. 2A). In both H460 and A549 cells, 24 h exposure to TFT alone induced the accumulation of cells in the S-phase while longer treatment, up to 72 h, was accompanied by an increase of cells in G2/M and a decrease in G1. This effect was most prominent in A549 cells. At the same time a strong rise in the percentage (up to around 35%) of death (sub-G1) cells was found for both cell lines. TRAIL alone mildly affected the cell cycle distribution of H460 cells with a small increase of cells in G1. This pattern was more pronounced in A549 cells. When TRAIL was combined with TFT, in H460 cells an increase in G2/M cells was seen although lower than found in TFT-treated cells. In A549 cells, the cell cycle profile after combined treatment resembled more that of TFT-treated cells, with increased S-phase after 24 h followed by a pronounced accumulation of G2/M cells. In addition, only a very small percentage of A549 cells was detected in the S-phase after 48 and 72 h of combined treatment.

Next, the treatments were examined for affecting the cell cycle regulatory proteins Chk1, Chk2, and Cdc25c. Chk1 and Chk2 are known to become phosphorylated after DNA damage resulting amongst others in inactivation of Cdc25c phosphatase by stimulating its proteasome-dependent degradation leading to G2/M and G1/S phase arrest [22]. TFT treatment induced strong Chk2 phosphorylation in a time-dependent manner, which correlated with a decrease in Cdc25c levels and G2/M arrest (Fig. 2A). Total Cdc25c levels decreased after 48 and 72 h treatment with TFT and even more rapidly after combination treatment reflecting its Chk-induced phosphorylation-dependent degradation. In H460

and A549 cells, exposure to TRAIL slightly increased the phosphorylation of Chk1, whereas Chk2 and Cdc25c phosphorylation levels remained similar (Fig. 2B) reflecting an increase of cells in the G1 phase (Fig. 2A).

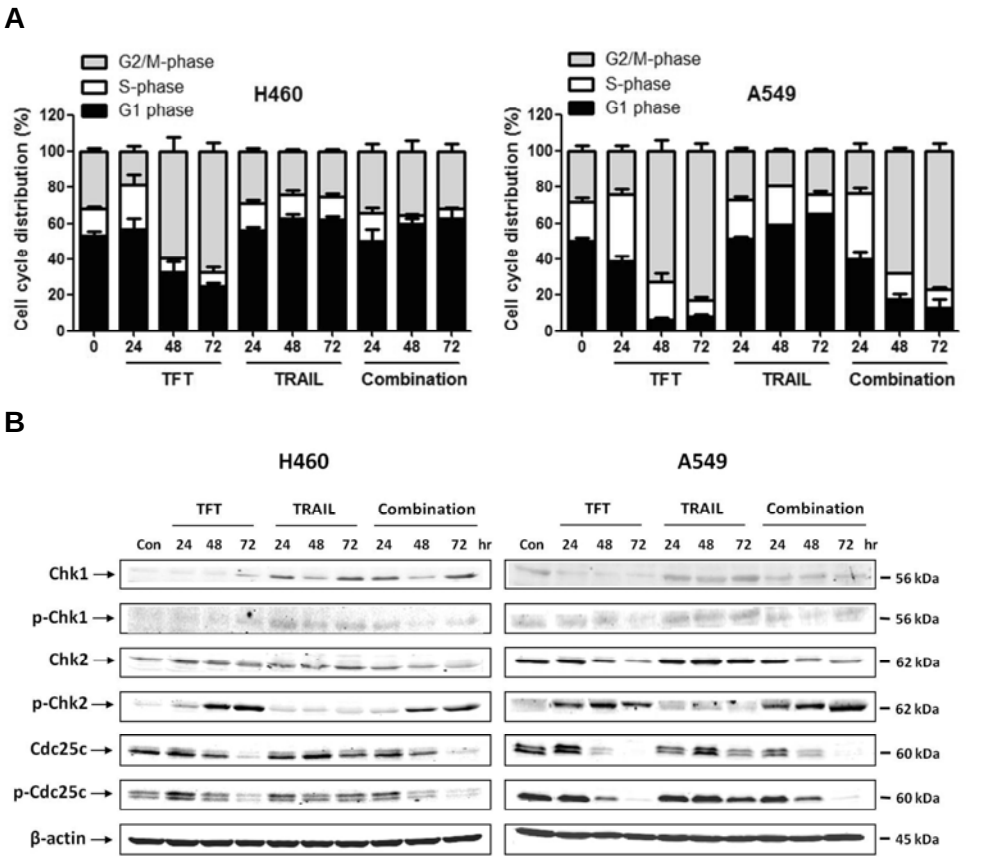

Figure 2. Effect of TRAIL, TFT and combined treatment on the cell cycle progression. (**A**) The percentages of cells in G1, S and G2/M phase are depicted after the indicated treatments in H460 and A549 cells. Values are means of three independent experiments ± SEM. After 24 h or 48 h incubation with TRAIL, medium was refreshed with drug free medium. (**B**) Western blots showing the expression and phosphorylation of cell cycle regulatory proteins Chk-1, Chk-2 and Cdc25c. Cells were exposed to 5 µM TFT, and 10 ng/ml TRAIL in H460, and 5 µM TFT, 150 ng/ml TRAIL in A549 or the combinations for 72 h.

Cell death activation by TFT and TRAIL

TFT triggered cell death in a time-dependent manner in H460 and A549 cells, whereas TRAIL killed H460 and not A549 cells as expected (Fig. 3A). In TRAIL resistant A549 cells the combination of TFT and TRAIL resulted in more cell death activation within 24 h that further increased in time. Since TFT alone induced cell death at exposure times longer than 24 h, the early cell killing effect in the combination was likely attributed to sensitized

TRAIL-dependent cell death. At 72 h, levels of dead cells were not higher than with TFT alone. This seems to contrast with the synergistic effect measured in the MTT assay. However, the sub-G1 level is an indication of the ratio between apoptotic and non-apoptotic cells, while the MTT assay also takes growth inhibition into account.

The contribution of caspase-dependent cell death was investigated by adding the broad-range caspase inhibitor zVAD to the cultures. This resulted in a partial inhibition of around 30 to 40% of TFT-induced cell death, indicating that caspase-independent cell death is a major contributor to cell death (Fig. 3B). TRAIL-induced apoptosis, as expected, was completely prevented by zVAD. In the first 24 h of combined treatment, the induction of cell death was completely inhibited by zVAD further indicating that at this time-point TFT enhanced or sensitized TRAIL-dependent cell death in both H460 and A549 cells. After another 48 h incubation with TFT alone, cell death was partially prevented by zVAD in line with the observations in TFT alone treated cells, suggesting that TFT-dependent cytotoxicity contributed to cell death at longer exposure times.

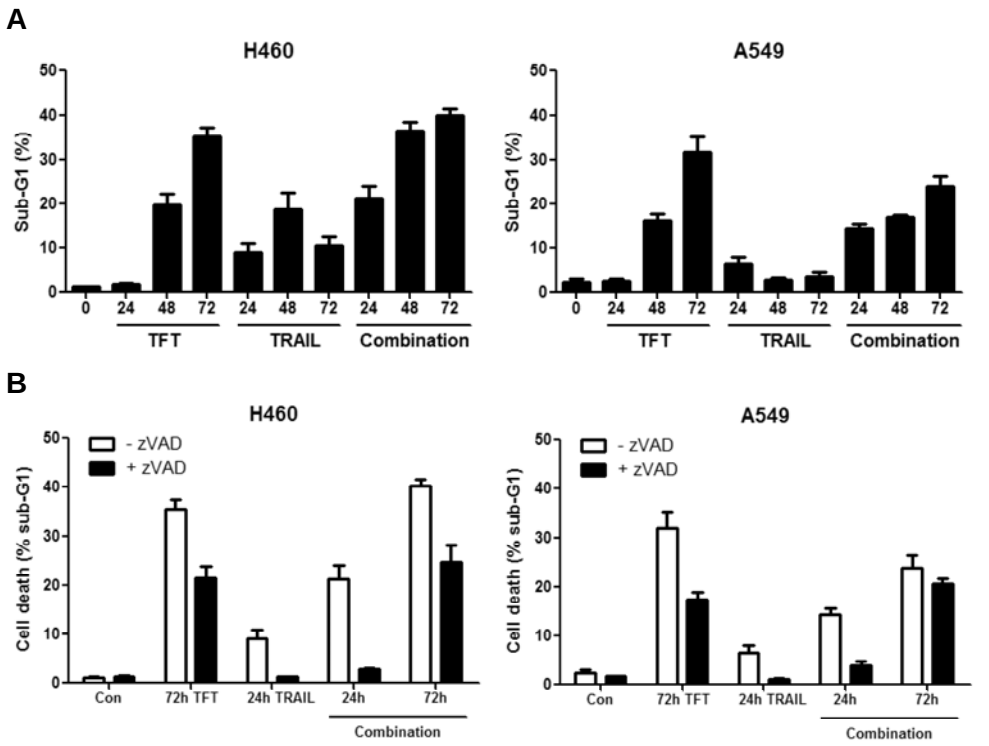

Figure 3. Caspase-dependent and -independent cell death activation. (**A**) TRAIL, TFT and the combination were examined for time-dependent cell death activation by determining the percentage of sub-G1 cells in PI-stained cells. (**B**) Comparison of cell death activation in the presence or absence of zVAD-fmk. Combined treatments of 24 and 48 h included a 24 h combination of TRAIL and TFT followed by 48 or 24 h period in drug free medium (DFM), respectively. TFT at 5 µM, TRAIL (10 ng/ml in H460), TRAIL (150 ng/ml in A549) were used for single and combined treatments. Values are means of three independent experiments ± SEM.

Caspase and cathepsin B activation by TRAIL and TFT

The effects of TFT and TRAIL on caspase-dependent apoptosis activation were analyzed in more detail by western blotting and fluorescent substrate-based activity assays (Fig. 4A and B). Cleavage of the downstream caspase substrate, PARP, an indicator of apoptotic cell death was also determined. Exposure of H460 and A549 cells to the IC_{50} concentration of TFT hardly resulted in cleavage of any of the tested caspases as indicated by no apparent decrease in the levels of procaspases and lack of detectable cleaved forms (Fig. 4A). This was in line with the unchanged caspase activity and the absence of PARP cleavage (Fig. 4A and B). In sensitive H460 cells, 24 h exposure to TRAIL induced caspase-8, -9 and -3 cleavage and activation. Resistant A549 cells showed low levels of caspase cleavage and activation as well as some cleavage of downstream PARP, however not sufficient to trigger an apoptotic response. When cells were exposed to combined TRAIL and TFT for 24 h, strong caspase cleavage was seen in both cell lines. Most notably caspase-3/7 and caspase-6/8 activity were stronger after combination treatment compared to either drug alone. PARP was almost completely cleaved in H460 cells. In conclusion, TFT did not trigger effective caspase activation, but it sensitized for TRAIL-induced caspase cleavage in A549 cells and enhanced caspase cleavage in H460 resulting in strong PARP cleavage and apoptosis.

A

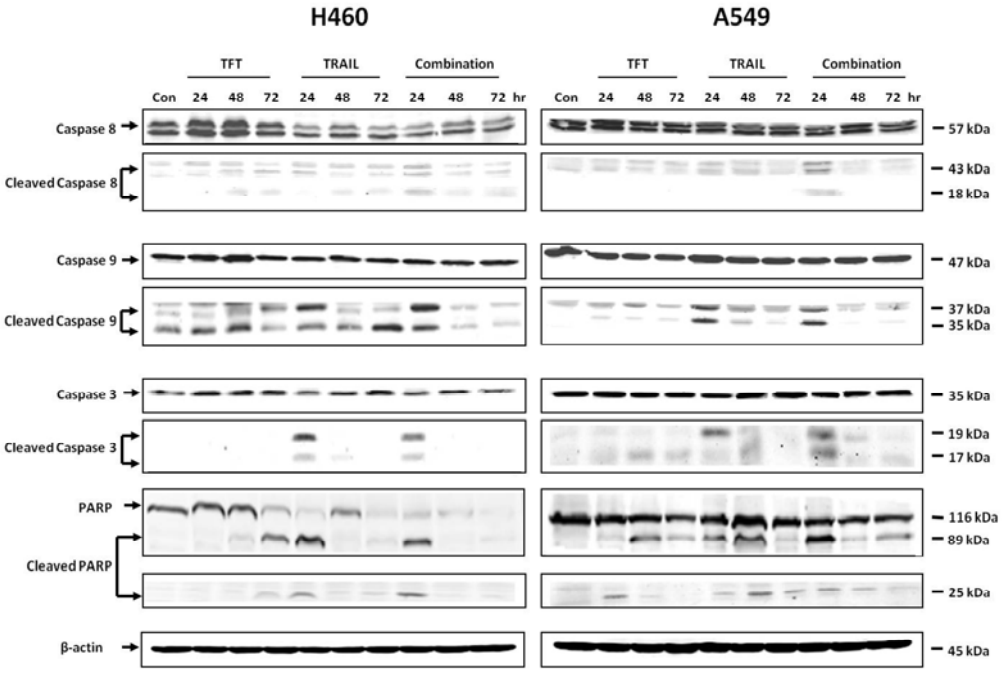

Figure 4. Caspase and cathepsin B activation in TRAIL- and TFT-treated H460 and A549 cells. (**A**) Western blot experiments showing caspase-8, -9, -3 and PARP cleavage. Procaspase and cleaved products are indicated.

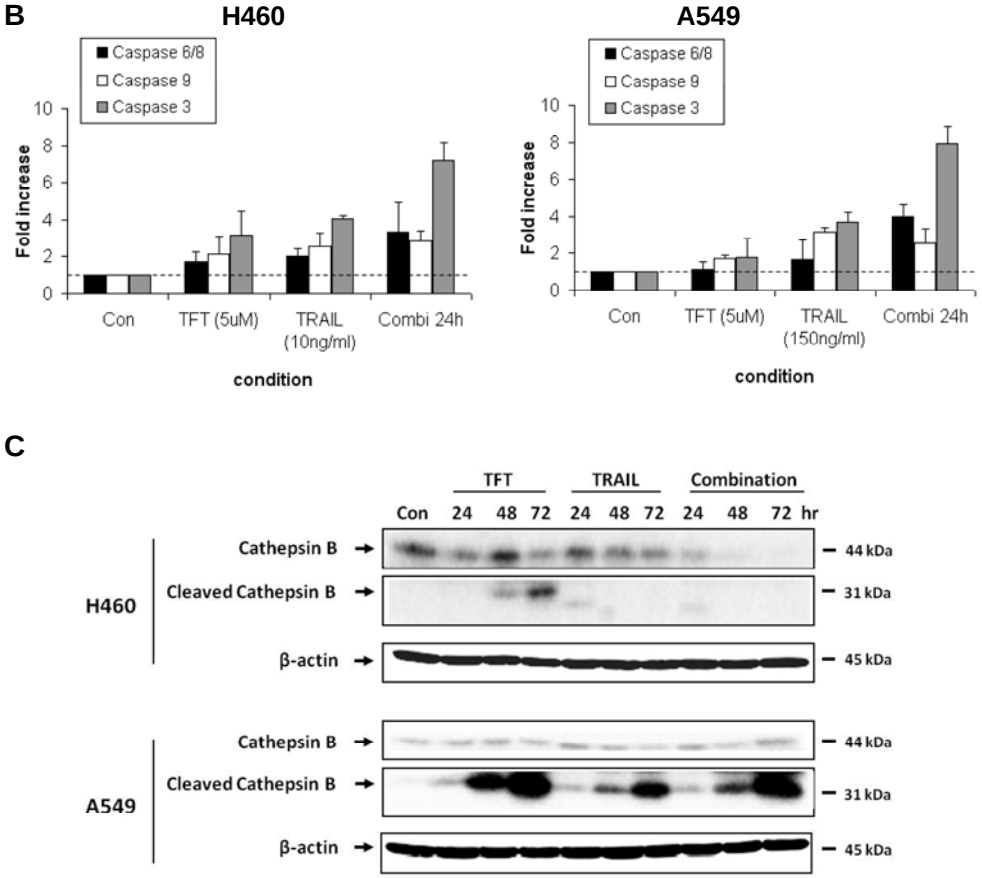

Figure 4 (continued). Caspase and cathepsin B activation in TRAIL- and TFT-treated H460 and A549 cells. (**B**) Fluorescent substrate-based caspase activity assays following 24 h incubation with TFT, TRAIL or the combination. Fold increase in activity of caspase-3/7, caspase-6/8 and caspase-9 are compared to untreated controls. TFT at 5 µM, 10 ng/ml TRAIL (H460) and 150 ng/ml TRAIL (A549). (**C**) Cathepsin B cleavage in H460 and A549 cells after TFT, TRAIL and combination treatment. Western blots showing pro-cathepsin B and cleaved products after exposing cells to 5 µM TFT, 10 ng/ml TRAIL (H460), 150 ng/ml TRAIL (A549) or the combinations for the indicated time points.

Since TFT-induced cell death was to a large extent caspase-independent a possible role of cathepsin B was investigated. Cathepsin B is expressed as an inactive pro-enzyme (43 kDa), which is cleaved into active cathepsin B (25 kDa or 31 kDa) upon lysosomal activation. TFT exposure induced cathepsin B cleavage at 48 and 72 h post-treatment (Fig. 4C). This cleavage is much stronger in A549 than H460 cells. Furthermore, TRAIL treatment increased cathepsin B cleavage progressively during time in A549 cells, whereas no cleavage was detected in H460 cells. When combining TRAIL and TFT, cathepsin B cleavage was also observed at later time points in A549 cells. In contrast, in H460 cells no cathepsin B cleavage is found after combined treatment. Finally, the effect of the

cathepsin B inhibitor, CA074-me, on TRAIL and TFT-induced cell death was examined. CA074-me had no effect on the level of cell death induction of any treatment, suggesting that cathepsin B was not directly involved in mediating TFT and/ or TRAIL-induced cell death (data not shown).

Effects of TFT on expression of TRAIL receptors and apoptosis regulatory proteins

The molecular mechanisms underlying the synergistic interaction between TFT and TRAIL were investigated. First, western blotting showed that TFT increased the levels of p53 within 24 h, which was also associated with p21/ WAF1 up-regulation in A549 and H460 cells (Fig. 5A). TFT reduced the expression of $cFLIP_L$, FLIPs was not detectable, and XIAP in both cell lines. Moreover, TFT increased the expression of pro-apoptotic Bax, whereas the expression of Bcl-2 and Bcl-XL did not alter (not shown). Second, the effect of TFT on the level of expression of the TRAIL receptors was determined by flow cytometry. As depicted in Fig. 5B, 24 h incubation of TFT increased the cell surface expression of mainly TRAIL-R2 and to a lesser extent TRAIL-R1 in both cell lines. As TFT increased the expression of p53, and it is known that p53 can upregulate the death receptors [23], we examined p53-dependancy of this up-regulation. A siRNA specifically directed against p53 strongly reduced p53 expression in H460 and A549 cells, whereas control siRNA had no effect when compared to untransfected cells (Fig. 5B). Down-regulation of p53 expression followed by TFT exposure resulted predominantly in reduced TRAIL-R2 up-regulation and mainly in A549 cells (Fig. 5C).

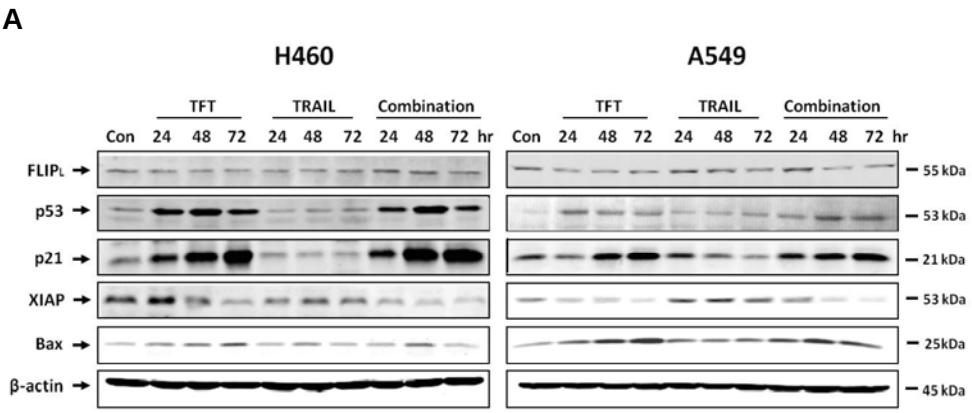

Figure 5. Mechanisms of TFT-dependent TRAIL sensitization. (A) Western blots showing the expression of $FLIP_L$, p53, p21, XIAP, and Bax after 24, 48 and 72 h exposure to TFT, TRAIL, and the combination. Representative blots are shown.

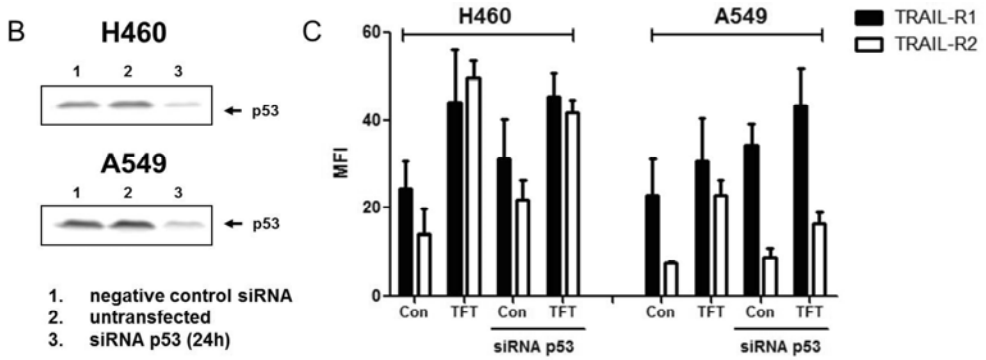

Figure 5 (continued). Mechanisms of TFT-dependent TRAIL sensitization. (**B**) Cells were transfected with negative control siRNA and p53 specific siRNA and p53 expression was determined by western blotting compared to non-transfected cells. (**C**) Effect of TFT on TRAIL-R1 and -R2 cell surface expression. Cells were exposed to 5 μM TFT for 24 h and TRAIL receptor expression was determined by FACS analysis. P53 was silenced with siRNA, incubating the cells for 24 h prior to TFT treatment. The graph depicts the changes in mean fluorescence intensity (MFI) of the TRAIL receptors compared to IgG1 isotype controls of at least two independent experiments.

DISCUSSION

Resistance of cancer cells to apoptosis is one of the causes underlying resistance to anti-cancer therapy. Sensitization of cancer cells to apoptosis could therefore be a valuable strategy to define new treatment options for cancer in particular when using agents that aim to directly activate apoptotic pathways [24]. The induction of apoptosis by TRAIL is essentially dependent on the activation of caspases. The present study shows that the novel thymidylate synthase inhibitor TFT can synergistically enhance TRAIL-induced apoptosis in NSCLC cells. TFT enhanced TRAIL-induced caspase-dependent apoptosis in TRAIL sensitive NSCLC cells and could restore TRAIL sensitivity in resistant NSCLC cells. However, the sequence of administration determined the synergistic effect of combined TFT and TRAIL treatment. Concurrent TRAIL/TFT exposure sensitized for TRAIL dependent apoptosis, whereas first TFT exposure followed by TRAIL treatment did not enhance apoptosis and even had antagonistic activity.

We identified a number of molecular mechanisms that are likely accountable for the sensitizing effect of TFT on TRAIL-induced apoptosis. TFT treatment resulted in the up-regulation of TRAIL-R2 and down-regulation of cFLIP, which is known to increase DISC formation and subsequent caspase-8 activation. This is in line with the observed enhancement of caspase-8 cleavage by TRAIL/TFT. TFT treatment also led to the accumulation of p53 and p21 known to mediate cell cycle arrest and activation of mitochondrial apoptosis. Furthermore, the TFT-mediated increase in Bax expression and a decrease in XIAP will also facilitate the activation of the mitochondrial apoptosis by TRAIL. It is likely that all the above mentioned pro-apoptotic effects of TFT are contributing to

the synergistic activity of TFT and TRAIL.

Chemotherapy and radiation are known to cause an enhancement of TRAIL receptor cell surface expression, mostly affecting TRAIL-R2 expression and to a lesser extent TRAIL-R1 [25]. We also found that TFT specifically enhances TRAIL-R2 expression and to a lesser extent that of TRAIL-R1. The TRAIL-R2 gene has transcription factor binding sites for p53 that mediate cytotoxic agent-dependent enhancement of expression [23]. In A549 cells, the up-regulation of TRAIL-R2 by TFT was indeed largely p53-dependent. In H460 cells the TFT-mediated increases in TRAIL receptor expression appeared not to be dependent on p53. Alternative mechanisms of TRAIL receptor up-regulation have been described such as by NF-κB [23].

Chemotherapeutics are known to affect cFLIP expression amongst others via transcriptional regulation by multiple factors and by posttranslational regulation involving rates of proteasomal degradation [8]. TFT downregulated $cFLIP_L$, a property that was also found for the well-studied nucleoside analogue 5-fluorouracil in colon cancer cells [26]. Accordingly we observed stronger caspase-8 cleavage after combined TRAIL/TFT treatment. However, Galligan and co-workers found no effect of 5-fluorouracil on TRAIL receptor expression independent of p53 status [26], in contrast to our observations with TFT. In another study, an increase of TRAIL receptor expression was reported for another nucleoside analogue, fludarabine, in chronic lymphocytic leukemia cells [27]. Comparable to TFT, fludarabine has been reported to decrease the expression of XIAP in chronic lymphocytic leukemia (CLL) cells [28]. Furthermore, the nucleoside analogs, fludarabine, cladribine and clofarabine promote cell death by alterations in MOMP causing cytochrome c release, as a consequence of Bax and Bak translocation to the mitochondria, in a p53-dependent manner [29]. Together this illustrates that available nucleoside analogues have different or overlapping TRAIL sensitizing properties, depending on the tumor type and cellular context. The property of TFT to enhance TRAIL-R2 expression may indicate that combined treatment with TRAIL-R2 agonists may have beneficial effects.

We also explored possible drug interactions at the level of cell cycle progression. TRAIL caused an accumulation of cells in the G1 phase. A small increase in phosphorylated Chk1 after TRAIL exposure may be responsible for this arrest. An increase of p-Chk1 upon TRAIL treatment has not been reported before. However, phosphorylation of Chk2 by TRAIL has been described in colon and cervix carcinoma cells [30]. TRAIL was found to induce a DNA damage response pathway in which also DNA-PK (DNA-dependent protein kinase) and ATM (ataxia telangiectasia) were activated leading to Chk2 phosphorylation. In a similar way this may lead to Chk1 phosphorylation by TRAIL as we observed in this study. Alternatively, TRAIL-induced accumulation of p21 (WAF1), may lead to cell cycle arrest as has been reported previously in human T-cells [31]. However, we did not detect a TRAIL-dependent increase in p21 levels. Combined treatment resulted in similar cell cycle effects compared to TFT alone, indicating that drug interactions at the level of cell cycle

regulation and checkpoint-dependent apoptosis activation are not likely to underlie the synergistic effects.

The mechanism of TFT-induced cell death was also further addressed in this study. Since cell death by TFT was largely caspase-independent we examined the possible involvement of the lysosomal protease cathepsin B in cell death activation. Previously, we found TFT as well as 5-fluorouracil to be able to activate cathepsin B in colon cancer cell lines and a variable contribution of cathepsin B cleavage to cell death activation was found [11]. Others have reported that TRAIL can induce apoptotic cell death in oral squamous cell carcinoma cells through activation of cathepsin B [32]. In the current study we also observed cleavage of cathepsin B induced by both TFT and TRAIL, in particular in A549 cells. However, inhibition of cathepsin B by a chemical inhibitor did not affect cell death activation. Thus, the more precise way by which TFT eradicates NSCLC cells remain to be identified. Regardless of this, the property of TFT to kill NSCLC tumor cells partially via caspase-independent mechanisms may be an advantage compared to other nucleoside analogues with respect to bypassing apoptosis-resistance mechanisms.

In conclusion, the present study demonstrates synergistic interactions between TFT and TRAIL in NSCLC cells. As TFT is also able to sensitize TRAIL-resistant NSCLC cells, this combination of TFT and TRAIL may offer a potential novel regime for NSCLC treatment.

Acknowledgements

This research was performed within the framework of project T3-112 of the Dutch Top Institute Pharma and partly supported by Taiho Pharmaceuticals, Tokushima, Japan.

Conflict of interest

Dr. Fukushima was employee of Taiho Pharmaceuticals.

Reference List

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007; 57(1):43-66.
2. Mountain CF. New prognostic factors in lung cancer. Biologic prophets of cancer cell aggression. Chest 1995; 108(1):246-254.
3. Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov 2008; 7(12):1001-1012.
4. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003; 22(53):8628-8633.
5. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004; 116(2):205-219.
6. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 2007; 6(7):2103-2112.
7. Pore MM, Hiltermann TJ, Kruyt FA. Targeting apoptosis pathways in lung cancer. Cancer Lett 2010.
8. Bagnoli M, Canevari S, Mezzanzanica D. Cellular FLICE-inhibitory protein (c-FLIP) signalling: a key regulator of receptor-mediated apoptosis in physiologic context and in cancer. Int J Biochem Cell Biol 2010; 42(2):210-213.
9. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
10. Temmink OH, Emura T, de Bruin M, Fukushima M, Peters GJ. Therapeutic potential of the dual-targeted TAS-102 formulation in the treatment of gastrointestinal malignancies. Cancer Sci 2007; 98(6):779-789.
11. Bijnsdorp IV, Peters GJ, Temmink OH, Fukushima M, Kruyt FA. Differential activation of cell death and autophagy results in an increased cytotoxic potential for trifluorothymidine compared to 5-fluorouracil in colon cancer cells. Int J Cancer 2010; 126(10):2457-2468.
12. Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. Clin Cancer Res 2005; 11(9):3155-3162.
13. Emura T, Murakami Y, Nakagawa F, Fukushima M, Kitazato K. A novel antimetabolite, TAS-102 retains its effect on FU-related resistant cancer cells. Int J Mol Med 2004; 13(4):545-549.
14. Overman MJ, Varadhachary G, Kopetz S, Thomas MB, Fukushima M, Kuwata K et al. Phase 1 study of TAS-102 administered once daily on a 5-day-per-week schedule in patients with solid tumors. Invest New Drugs 2008; 26(5):445-454.
15. Yoshino T, Mizunuma N, Yamazaki K, Nishina T, Komatsu Y, Baba H et al. TAS-102 monotherapy for pretreated metastatic colorectal cancer: a double-blind, randomised, placebo-controlled phase 2 trial. Lancet Oncol 2012; 13(10):993-1001.
16. Keepers YP, Pizao PE, Peters GJ, van Ark-Otte J, Winograd B, Pinedo HM. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. Eur J Cancer 1991; 27(7):897-900.
17. Bijnsdorp IV, Kruyt FA, Gokoel S, Fukushima M, Peters GJ. Synergistic interaction between trifluorothymidine and docetaxel is sequence dependent. Cancer Sci 2008; 99(11):2302-2308.
18. Bijnsdorp IV, Capriotti F, Kruyt FA, Losekoot N, Fukushima M, Griffioen AW et al. Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors. Br J Cancer 2011; 104(7):1185-1192.
19. Mathews ST, Plaisance EP, Kim T. Imaging systems for westerns: chemiluminescence vs. infrared detection. Methods Mol Biol 2009; 536:499-513.
20. Koyama S, Koike N, Adachi S. Expression of TNF-related apoptosis-inducing ligand (TRAIL) and its receptors in gastric carcinoma and tumor-infiltrating lymphocytes: a possible

mechanism of immune evasion of the tumor. J Cancer Res Clin Oncol 2002; 128(2):73-79.
21. Duiker EW, Meijer A, van der Bilt AR, Meersma GJ, Kooi N, van der Zee AG et al. Drug-induced caspase 8 up-regulation sensitises cisplatin-resistant ovarian carcinoma cells to rhTRAIL-induced apoptosis. Br J Cancer 2011; 104(8):1278-1287.
22. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature 2004; 432(7015):316-323.
23. Mendoza FJ, Ishdorj G, Hu X, Gibson SB. Death receptor-4 (DR4) expression is regulated by transcription factor NF-kappaB in response to etoposide treatment. Apoptosis 2008; 13(6):756-770.
24. Frese S, Frese-Schaper M, Andres AC, Miescher D, Zumkehr B, Schmid RA. Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5. Cancer Res 2006; 66(11):5867-5874.
25. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. Biochim Biophys Acta 2010; 1805(2):123-140.
26. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther 2005; 4(12):2026-2036.
27. Johnston JB, Kabore AF, Strutinsky J, Hu X, Paul JT, Kropp DM et al. Role of the TRAIL/APO2-L death receptors in chlorambucil- and fludarabine-induced apoptosis in chronic lymphocytic leukemia. Oncogene 2003; 22(51):8356-8369.
28. Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S et al. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood 1998; 91(9):3379-3389.
29. Ewald B, Sampath D, Plunkett W. Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene 2008; 27(50):6522-6537.
30. Solier S, Sordet O, Kohn KW, Pommier Y. Death receptor-induced activation of the Chk2- and histone H2AX-associated DNA damage response pathways. Mol Cell Biol 2009; 29(1):68-82.
31. Bosque A, Aguilo JI, del Rey M, Paz-Artal E, Allende LM, Naval J et al. Cell cycle regulation by FasL and Apo2L/TRAIL in human T-cell blasts. Implications for autoimmune lymphoproliferative syndromes. J Leukoc Biol 2008; 84(2):488-498.
32. Nagaraj NS, Vigneswaran N, Zacharias W. Cathepsin B mediates TRAIL-induced apoptosis in oral cancer cells. J Cancer Res Clin Oncol 2006; 132(3):171-183.

Chapter 7

Accumulation of thymidine-derived sugars in thymidine phosphorylase overexpressing cells.

Irene V. Bijnsdorp, Kaamar Azijli, Erwin E. Jansen, Mirjam M. Wamelink, Carel Jakobs, Eduard A. Struys, Masakazu Fukushima, Frank A.E. Kruyt, Godefridus J. Peters

Biochemical Pharmacology, 2010; 80(6):786-92

ABSTRACT

Thymidine phosphorylase (TP) is often overexpressed in cancer and potentially plays a role in the stimulation of angiogenesis. The exact mechanism of angiogenesis induction is unclear, but is postulated to be related to thymidine-derived sugars. TP catalyzes the conversion of thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P), which can be converted to dR-5-P, glyceraldehyde-3-phosphate (G3P) or deoxyribose (dR). However, it is unclear which sugar accumulates in this reaction. Therefore, in the TP overexpressing Colo320 TP1 and RT112/TP cells we determined by LC-MS/MS which sugars accumulated, their subcellular localization (using ^{3}H-TdR) and whether dR was secreted from the cells. In both TP-overexpressing cell lines, dR-1-P and dR-5-P accumulated intracellularly at high levels and dR was secreted extensively by the cells. A specific inhibitor of TP completely blocked TdR conversion, and thus no sugars were formed. To examine whether these sugars may be used for the production of angiogenic factors or other products, we determined with ^{3}H-TdR in which subcellular location these sugars accumulated. TdR-derived sugars accumulated in the cytoskeleton and to some extent in the cell membrane, while incorporation into the DNA was responsible for trapping in the nucleus. In conclusion, various metabolic routes were entered, of which the TdR-derived sugars accumulated in the cytoskeleton and membrane. Future studies should focus on which exact metabolic pathway is involved in the induction of angiogenesis.

Key Words: Thymidine phosphorylase, angiogenesis, deoxyribose, thymidine phosphorylase inhibitor

INTRODUCTION

The platelet derived endothelial cell growth factor (PD-ECGF) or thymidine phosphorylase (TP) is often overexpressed in human cancers, which has been related to a higher microvessel density, a higher tumor stage and the induction of metastasis [1-4]. In various *in vitro* studies, TP had a chemotactic effect on endothelial cells [5-8]. *In vivo*, TP induced angiogenesis using various cancer cell lines that were transfected with TP [9;10]. The role of TP in angiogenesis seems evident, but its exact mechanism is still unknown and it is postulated to be related to the sugars that are formed from thymidine (TdR) degradation by TP.

Deoxy-α-D-ribose-1-phosphate (dR-1-P) and thymine are the first products that are formed by the phosphorylysis of TdR by TP (Fig. 1) [11]. After its formation, dR-1-P rapidly disappears, possibly by conversion to 2-deoxy-D-ribose (dR) or 2-deoxyribose-5-phosphate (dR-5-P) [12]. dR-5-P can be converted to glyceraldehyde-3-phosphate (G3P), which can enter the glycolytic or pentose phosphate pathway. dR can form advanced glycation end products (AGE) or reactive oxygen species (ROS) by Schiff base reactions [13]. The Schiff base is formed from either dR or dR-5-P, which can be coupled to an intracellular protein by nonenzymatic condensation between the sugar's aldehyde group and a lysine residue. This Schiff base can subsequently rearrange to an Amadori product, such as an α-hydroxyketone, which may also form an enediol intermediate [14]. These unstable intermediates react via non-enzymatic reactions to form AGEs. During these reactions, specifically in the transition metal-catalyzed auto-oxidation, free radicals are produced [15-17]. The formation of AGE from these reactions could be responsible for the angiogenic activity of TP [13].

The enzymatic activity of TP is indispensable for the angiogenic effect, since a competitive inhibitor of TP could block the angiogenic effect [18]. Therefore, substrate and metabolites from the reaction are often used for studying the angiogenic effect of TP [19]. The metabolite dR has previously shown angiogenic activity [5;10] and could elongate the sprouting in the aortic ring assay [19]. In addition, dR induced endothelial cell migration in various studies [7;8]. The association between TP activity and angiogenesis is based on the potential accumulation of TdR-derived sugars. However, little is known about the cellular metabolism of dR-1-P. Although dR is considered the main factor causing the angiogenic switch, the real identity and extent of formation of TdR-derived sugars remains unclear. dR-1-P rapidly disappears after its formation and its conversion could be reduced by addition of dR-5-P [11]. The aim of the present study was to characterize the specific sugars that can be formed by the TP reaction, their subcellular localization and whether they are secreted by the cancer cells, enabling endothelial cells to migrate and invade towards tumor sites.

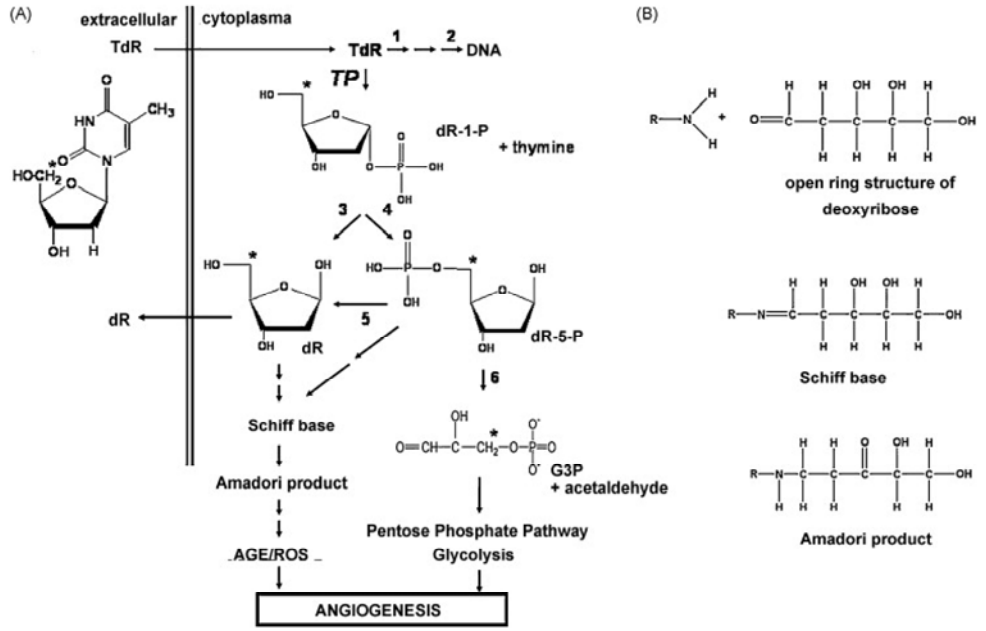

Figure 1. Schematic overview of the conversion of TdR to TdR-related sugars and possible pathways which can stimulate angiogenesis. (**A**) TdR enters the cell, after which it is converted to thymine and deoxyribose-1-phosphate (dR-1-P). dR-1-P can be further converted to dR or dR-5-P. dR-5-P can enter the pentose phosphate pathway or glycolysis, stimulating the metabolism of the cells, possibly activating angiogenesis. dR is secreted by the cells, after which it can attract endothelial cells and/ or be taken up by the endothelial cells, stimulating angiogenesis. Both dR and dR-5-P can undergo Schiff base reactions, first by opening the ring structure of the sugar by anomerization, after which it can undergo a Schiff base reaction where an Amadori product is formed. Subsequently, reactive oxygen species (ROS) are formed, or advanced glycation end products (AGE). Moieties at which [5'-^{3}H]-TdR was labeled and the TdR-derived sugars are indicated with *. TP: thymidine phosphorylase 1. thymidine kinase (TK); 2. DNA polymerase; 3. a phosphatase; 4. phospho-pentomutase; 6. deoxyribosephospatealdolase. (**B**) Structural formulae of the open ring structure of dR (2-deoxy-D-ribose), a Schiff base and an Amadori product. The open chain aldehyde glycates protein by a non-enzymatic condensation of the sugar to form a Schiff base, which rearranges to form an α-hydroxyketone Amadori product. dR-5-P can undergo the similar reaction. R= protein (see also ref [13]).

MATERIALS & METHODS

Cell culture and chemicals

The human colon carcinoma cell line Colo320 TP1 was a variant of Colo320 (obtained from the ATCC), transfected with TP, as described previously [20]. RT112/TP was a kind gift of Dr. Bicknell (Oxford, UK). Cells were cultured as monolayers in DMEM, supplemented with 10% heat inactivated FCS and 20 mM Hepes in 25 cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany). Cells were maintained in a humidified 5% CO_2 atmosphere at

37°C. TPI was provided by Taiho Pharmaceutical, Co., Ltd. (Tokushima, Japan). TdR, thymine, dR, dR-1-P, dR, dR-5-P and G3P were obtained from Sigma Aldrich Chemicals (Zwijndrecht, the Netherlands). These chemicals were dissolved in PBS in stock solutions of 1-20 mM and stored at -20 °C. $^{13}C_6$-glucose-6-phosphate (prepared as previously described [21]) and $^{13}C_2$-xylulose were obtained from Omicron biochemicals (South Bend, IN, USA). Ethoxyamine was obtained from Acros (Geel, Belgium) and pyridine from Merck (Darmstadt, Germany). 5'-^{3}H-TdR (specific activity of 18.8 mCi/mmol, conc. 12.9 µg/ml) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). The exact moiety where the sugar of the TdR is labelled is indicated in Fig. 1. The thymidine phosphorylase antibody was purchased from R&D systems Inc. (Minneapolis, MN USA) and the thymidine kinase antibody was purchased from QED Bioscience (San Diego, CA, USA). The secondary antibody, goat-α-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) was obtained from Westburg (Leusden, the Netherlands).

Treatment of the cells

For measurement of TdR and its sugars, Colo320TP1 and RT112/TP cells were seeded at 2x10^6 cells/flask. After 48 h, cells were exposed to 100 µM TdR for 0 min, 15 min, 30 min and 60 min. TPI (10 µM) was added 24 h prior to TdR exposure, after which samples were incubated for 60 min. After treatment, cells were trypsinized, washed in PBS and centrifuged for 5 min at 323 g at room temperature (RT). The medium and the cell pellets were stored separately at -80°C until sample preparation.

Measurement of TP enzymatic activity and of TdR and thymine

HPLC measurement of TP enzymatic activity was performed as described previously [22] and is based on the detection of both TdR and thymine [23]. After incubation with thymidine, 25 µl 80% trichloroacetic acid (TCA) was added to the samples, which were left on ice for 20 min. Subsequently, samples were centrifuged at 14000 g at 4°C for 10 min. The supernatant was transferred to a new vial, and the pH was neutralized. Samples were mixed and centrifuged for 1 min at 14000 g. The upper aqueous layer was used for analysis of TdR and thymine by HPLC analysis for nucleosides with UV detection as described previously [23].

Measurement of sugar-phosphates

For sample preparation, the cell pellet was dissolved in 500 µl MQ and subsequently samples were sonificated 3 x for 3 seconds on ice. 5 µM $^{13}C_6$-glucose-6-phosphate was used as an internal standard. The samples were centrifuged through a filter with 10 kDa cut off (Ultracel YM-10; #42407; Amicon Micron, Millipore, Billerica**,** MA, USA). Subsequently, the samples were injected into the LC-MS/MS as described previously [24]. The amount of sugars was expressed based on the total protein content of each sample

that was measured before centrifugation through the 10 kDa cut off filter. Protein concentration was determined using the BioRad Protein assay (#500-0006; Bio-Rad Laboratories, Veenendaal, The Netherlands) according to manufacture's instruction.

Measurement of dR

For preparation of the samples for the dR measurement, the cell pellet was dissolved in 150 µL distilled water. $^{13}C_2$-xylulose was used as internal standard. 17 µl of 20 µM $^{13}C_2$-xylulose was added to 50 µl of the sample. To deproteinize the sample, 200 µl methanol was added. After 15 min, the sample was centrifuged for 10 min at 10 000 g at 4 °C. Following this, the supernatant was transferred to a new vial and evaporated to dryness under a slight stream of nitrogen. Ethoxime derivatives of dR were formed by treating the residue with 2 mg ethoxyamine in 100 µl pyridine at 60 °C for 30 min. After cooling down to RT, the hydroxygroups were acetylated by adding 100 µl acetic anhydride at 80°C for 60 min. This solution was evaporated to dryness and the residue was redissolved in ethylacetate. 1-2 µl was injected into the GC-MS operating under positive chemical ionization in the single ion monitoring mode.

Separation of cell compartments

*C*olo320 TP1 and RT112/TP cells ($1.5x10^6$ cells) were exposed to 200 µM TdR (hot:cold (1:21) of which the batch of [5'-^{3}H]-TdR was mixed with 1 mM unlabeled TdR). After incubation for 1, 6 or 24 h or 1 h plus 24 h [5'-^{3}H]-TdR-free medium at 37 °C, cell fractions were separated using a ProteoExtract® Subcellular Proteome Extraction Kit according to manufacture's instructions (Calbiochem, San Diego, CA). Of every cell fraction, including from the medium above the cells after the designated incubation times, 5 µl was counted. To determine to which fraction (protein or non-protein fraction) secreted [5'-^{3}H]-TdR-derived metabolites accumulated, 100 µl of the medium above the cells after the retention was precipitated with 60 µl 35% TCA for 20 min on ice. Subsequently, samples were centrifuged for 5 min at 300 g at 4°C and 5 µl of the supernatant (non-protein fraction) was counted. In addition, the pellet (protein fraction) was recovered in 200 µl MQ, of which 5 µl was counted.

Western blotting

Colo320, Colo320 TP1, RT112 and RT112/TP cells were washed twice with ice-cold PBS and lysed in lysis buffer (Cell Signalling Technology Inc., Denver, USA). Cell lysates were scraped, transferred into a vial and centrifuged at 11 000 g at 4 °C for 10 min. Supernatants were transferred to a new vial and protein amounts were determined by the Bio-Rad assay, according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the Netherlands). From each condition 30 µg of protein was separated on a 10% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes

(Millipore Immobilon^{TM} –FL PVDF, 0.45 µm). Subsequently, the membranes were blocked for 1 h at room temperature (RT) in Odyssey blocking buffer (Odyssey blocking buffer #927-40003, Westburg, Leusden, The Netherlands) and incubated overnight at 4°C with the primary antibodies (dilution 1:1000-10000 in Odyssey blocking buffer 1:1 diluted with PBS-T (PBS with 0.05% Tween-20). The membrane was washed 5 times in PBS-T and incubated with the secondary antibodies (1:10000) for 1 h at RT in the dark. After incubation, the membrane was washed in PBS-T and followed by 5 min washing in PBS without Tween-20 to decrease the background signal. Subsequently, the bands were scanned using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, Nebraska USA), 0 mm offset, 84 µm resolution and with high quality [25].

Statistical analysis

Potential differences between controls and TdR exposed cells were evaluated using the two-tailed Student's *t*-test for paired data. Changes were considered significantly different when $p<0.05$.

RESULTS

TdR conversion to thymine

In order to determine the extent of TdR conversion, TdR and thymine levels were measured, after incubation with 100 µM TdR for 15, 30 and 60 min. As expected, the parental Colo320 and RT112 cells did not or hardly convert TdR to thymine, since these cells do not or hardly express TP (Fig. 2) [20]. TP was highly expressed in both Colo320 TP1 and RT112/TP cells (Fig. 2), while no band could be detected in Colo320 parental cells and a very faint band was found in RT112 cells. Colo320 and RT112 cells converted thymidine at a rate of 0 ±0 and 3 ±0.04 nmol/ million cells/h, respectively. Colo320 TP1 and RT112/TP converted thymidine at a rate of 518 ±40 nmol/million cells/h and 418 ± 34 nmol/million cells/h (Fig. 3A). After 1 h incubation of intact cells with TdR, the extracellular thymine concentrations were 24 and 30 µM, respectively. The TK protein, which activates TdR for DNA synthesis, was similarly expressed in the TP-transfected cells (data not shown).

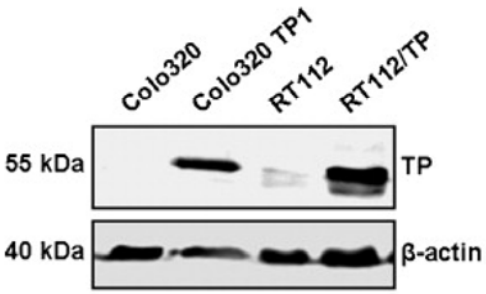

Figure 2. Western blot showing the expression levels of thymidine phosphorylase in Colo320, Colo320 TP1, RT112 and RT112/TP cells.

Intracellular accumulation of dR-5-P and dR-

TP degrades TdR into one molecule of thymine and one dR-1-P, which can be metabolized to other sugars (Fig. 1). We measured the intracellular levels of the potential metabolites dR-1-P, dR-5-P, dR and G3P in Colo320, RT112 and the transfected variants, Colo320 TP1 and RT112/TP. In the cells that did not express TP, no sugars accumulated at all (data not shown), which is in agreement with the absence of thymine formation. In general, the sugars accumulated to a much higher extent in RT112/TP cells, compared to Colo320 TP1 cells (Fig. 3A and 3B). dR-1-P increased rapidly, and was about 10 fold higher in RT112/TP cells compared to Colo320 TP1 cells. dR-1-P was rapidly metabolized to dR-5-P and dR. In both Colo320TP1 and RT112/TP cells, dR-1-P was the major intracellular metabolite (Fig. 3B). In RT112/TP cells, the accumulation of dR-5-P was lower than the level of dR-1-P. dR accumulated intracellularly to a low extent in Colo320TP1 cells and was only 4 fold increased compared to the control dR level. In RT112/TP cells, dR hardly accumulated. In

Colo320 TP1 cells, G3P increased only at very low levels, with a 2 fold increase compared to the control. G3P did not accumulate in RT112/TP cells.

Addition of TPI alone to the cultures did not alter the production of the tested sugars. TPI completely blocked the conversion of TdR in Colo320 TP1 and RT112/TP cells (Fig. 3A), which is in agreement with the formation of the sugars, which were not formed at all (Fig. 3A and 3B).

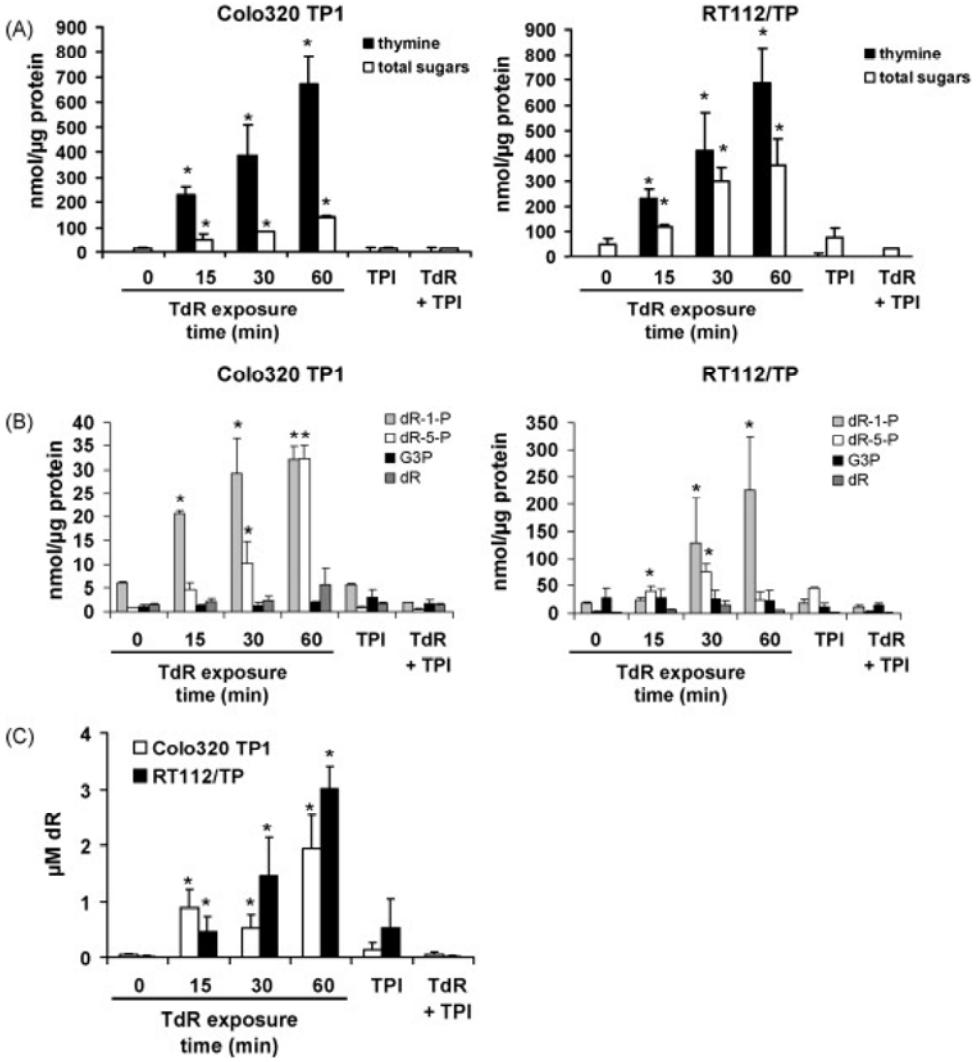

Figure 3. The accumulation of sugars after conversion of TdR by thymidine phosphorylase. (**A**) total levels of thymine and total levels of measured sugars. (**B**) Measurement of the levels of the various TdR-related sugars. (**C**) Measurement of the levels of dR that was secreted by the cells after degradation of TdR. All values represent means of three independent experiments ± SEM. Significant differences compared to the control levels are indicated * $P < 0.05$.

dR is secreted from the cells

dR is an important angiogenic sugar that can be secreted by the cells [6;13]. Therefore, we determined the level of dR in the medium. dR-1-P and dR-5-P can not cross the membrane, because of the negative charge of the phosphate group on these sugars. In the medium above Colo320 TP1 and RT112/TP cells, dR increased in time (Fig. 3C) and was found at higher levels than the other sugars intracellularly (Table 1). Of the total thymine detected, 10 and 13 % was measured as extracellular dR after 1 h by Colo320 TP1 and RT112/TP cells, respectively (Table 1). TPI alone did not have any effect on the intracellular levels of dR, while TPI prevented dR from being formed (Fig. 3C).

Thymidine-derived sugars and accumulation levels

Since the thymine levels are representative for the total amount of sugars that are formed, we compared each value with the amount of thymine (in which thymine was set at 100 % for each time point) (Table 1). In Colo320 TP1 cells, the total amount of measured sugars was much lower than that of RT112/TP cells (Fig. 3A). In Colo320 TP1 cells, about 20% of the total TdR-derived sugars presented intracellularly as dR-1-P, dR-5-P, dR and G3P and extracellular dR. This did not increase after a longer exposure time to TdR, indicating that the sugars are rapidly metabolized to other products. In RT112/TP cells, about 50 % of the TdR-derived sugars were measured (Table 1), indicating a slower metabolism of these sugars into other products compared to that in Colo320 TP1 cells.

Table 1 | Percentages of TdR related sugars compared to the total converted TdR.

	Colo320TP1			RT112/TP		
	15	30	60	15	30	60
dR-1-P	8.9 ± 0.4*	7.6 ± 2.7*	4.8 ± 0.6*	10.7 ± 0.9*	30.8 ± 2.7*	32.9 ± 2.6*
dR-5-P	1.9 ± 0.9	2.6 ± 1.7*	4.8 ± 0.6*	17.3 ± 3.5*	17.9 ± 2.9*	3.4 ± 0.9
G3P	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	12.2 ± 4.0	6.2 ± 1.5	3.4 ± 1.4
dR	0.9 ± 0.4	0.6 ± 0.3	0.8 ± 0.7	2.1 ± 0.7	3.4 ± 0.9	0.7 ± 0.1
Extracellular dR	10.8 ± 2.4*	10.4 ± 2.2*	10.0 ± 0.3*	10.3 ± 1.4*	12.8 ± 0.5*	13.0 ± 0.1*
Total sugars in relation to thymine	23.2 ± 2.5*	21.5 ± 4.1*	20.7 ± 1.7*	52.5 ± 0.2*	71.2 ± 4.5*	53.5 ± 4.1*
Total thymine	100	100	100	100	100	100

Values were calculated relative to the amount of thymine formed and represent means (%) of three independent experiments ± SEM. Significant differences compared to the control levels are indicated. * $P < 0.05$.

Accumulation in the cytoskeleton and nucleus

In order to determine in which cellular compartment the converted products of TdR accumulated, Colo320 TP1 and RT112/TP cells were exposed to [5'-^{3}H]-TdR for different time periods. Subsequently, subcellular cell fractions were separated and the amount of radioactivity was determined in each compartment (Fig. 4).

In Colo320 TP1 cells, the level of [5'-^{3}H]-products in the cytosolic compartment decreased in time, until it was hardly detectable after 24 h. Products in the membrane fraction increased to some extent after 6 h, while after 24 h hardly any radioactivity was

detectable in this fraction. In the nuclear fraction, the radioactivity was increased after 24 h, which was about 55% of the total intracellular radioactivity. This is possibly in part due to metabolic activation of TdR by thymidine kinase, resulting in incorporations into the DNA and nuclear trapping. Interestingly, TdR-derived radioactivity was present in the cytoskeletal protein fraction, which increased in time up to about 45% after 24 h.

In RT112/TP cells, the radioactivity in the cytosolic fraction decreased in time, with about 45% of [5'-^{3}H]-products still detectable after 24 h (Fig. 4). This is in agreement with the lower metabolism of the sugars in these cells, as measured by LC-MS/MS (Table 1). Comparable to Colo320 TP1 cells, the radioactive products in the nuclear fraction increased in RT112/TP cells, although at lower levels; up to 25% after 24 h. Both the membrane and cytoskeleton fraction increased in time, with a maximum of about 25% and 10% after 24 h incubation, respectively.

When the [5'-^{3}H]-TdR was washed away following 1 h incubation, the radioactivity was found to a high extent in the membrane (9.6 and 13.6%) and cytoskeletal (73.5 and 39.4%) compartments of both Colo320 TP1 and RT112/TP cells, respectively (data not shown). Hardly any of the radioactive label retained in the nuclear fraction, which may be related to a lack of activation of TdR by TK within 1 h exposure. In summary, the TdR-sugars are used in the cytoskeletal and membrane, which may possibly be related to protein-glycation.

Secreted products are in the non-protein fraction

To determine whether products were secreted after conversion of TdR, Colo320 TP1 and RT112/TP cells were exposed to [5'-^{3}H]-TdR for 1 h, after which the medium was refreshed and cells were washed to remove the excess of [5'-^{3}H]-TdR. The amounts of secreted products were determined by analyzing the amount of radioactivity in the medium fraction. Both Colo320 TP1 and RT112/TP cells secreted radioactive products, with higher levels for RT112/TP (24%) than for Colo320 TP1(15%) cells.

To analyze whether the secreted products were in the protein or in the non-protein fraction, proteins were precipitated by adding TCA (Fig. 4B). In both Colo320 TP1 and RT112/TP cells, the largest part of the secreted radioactive products was found in the non-protein fraction. This indicates that the sugars may not be used to a high extent for protein-glycation of secreted proteins, but may be more important for processes intracellularly.

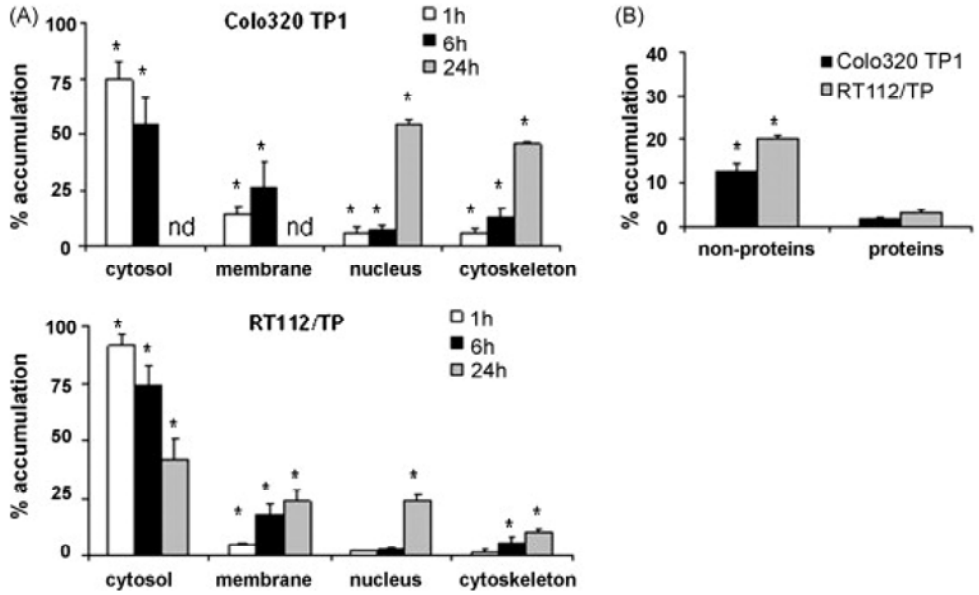

Figure 4. Accumulation of TdR-related sugars. (**A**) Accumulation in subcellular compartments of the cytosol, membrane, nucleus and cytoskeleton. Values represent means of three independent experiments ± SEM. nd = not detectable. (**B**) Percentage of secreted TdR-related sugars in the medium above the cells, relative to the total radioactivity intra- and extracellular. Cells were exposed for 1 h to TdR after which the medium was refreshed and incubated for another 24 h. Subsequently the medium was analyzed for TdR-related sugars total, in the protein and in the non-protein fraction. Values are mean % of total formed thymine of 3 independent experiments ± SEM.

DISCUSSION

In this study we describe TdR phosphorylysis to dR-1-P, which is rapidly followed by its isomerization to dR-5-P or degradation to dR of which the latter was extensively secreted. To our knowledge, the formation of these sugars using human (eukaryotic) cells has not been described earlier. We observed different levels of accumulation of TdR-derived sugars in the two cell types. In addition, the converted TdR was found in the cytoskeleton and to some extent in the cell membrane.

Previously we demonstrated that dR-1-P formed from TdR rapidly disappeared from Colo320 TP1 cells [11]. dR-1-P can be isomerized to dR-5-P mediated by phosphopentomutase (E.C. 5.4.2.7) and is degraded to dR by a phosphatase [10]. dR-5-P can be split into G3P plus acetaldehyde by deoxyriboaldolase (E.C. 4.1.2.4) [12;26]. The formed acetaldehyde may be converted into acetyl-CoA by aldehyde oxidase and acetyl-CoA synthetase [26]. G3P can enter several metabolic pathways, including the glycolytic and the pentose phosphate pathway, and possibly therefore did not accumulate in the cells in our study. Initially, these steps were only described in *e.g. Bacillus* ceresus [27]. Enzymatic activity needed for this pathway was also identified in epithelial anmoitic WISH cells [12]. The sugars may also be substrate in the formation of AGE (unpublished data), which may be used for protein glycation, e.g. adding sugar moieties to proteins by non-enzymatic reactions [16;28]. However, whether protein glycation of AGEs are formed in the cells used in our study, remains to be identified. Brown et al. previously reported a possible Schiff base reaction in RT112 cells, and demonstrated the presence of reactive oxygen species by expression levels of the oxidative stress marker heme oxygenase-1 (HO-1) [29].

dR-1-P can also be formed by conversion of purine deoxynucleosides by purine nucleoside phosphorylase (PNP) [30]. Therefore theoretically, PNP may also be an angiogenic enzyme. However, to the best of our knowledge, a role for PNP in angiogenesis has never been described before. Important differences between TP and PNP involve their function: TP overexpression in tumor sites has clearly been related with angiogenesis [1], while PNP is involved in immune function [31;32]. In addition, PNP is hardly overexpressed in cancer [31-33], making it less likely to be an angiogenic enzyme. It might be possible that other products than the sugars are involved in angiogenesis as well, such as β-Amino-iso-butyric acid, which is another downstream metabolite of thymine [19]. However, several enzymes involved in thymine catabolism (e.g. DPD and β-ureidopropionase) are usually low or not detectable in many tumors [34-36]. In our and other panels of colon cancer cell lines, DPD activity was low to not detectable [37;38], in contrast to pancreatic and breast cancer [37;39;40]. Therefore, it is unlikely that β-Amino-iso-butyric acid is involved in the angiogenic activity of TP.

In the present study, we used cancer cells to determine the sugar metabolism in order to stimulate angiogenesis. TP expression in endothelial cells may also be important for their

angiogenic properties. However, TP expression is very low in endothelial cells [41;42]. In addition, TP expression is often reported to be in either cancer cells or other tumor stromal cells such as fibroblasts. The angiogenic role of TP is possibly more related to the secretion of angiogenic factors (i.e. dR or chemoattractants such as IL-8) by cancer cells, rather than an autocrine stimulation by endothelial cells themselves. Therefore, the model that we used in the present study was intended to reflect the stimulation of angiogenesis by cancer cells.

Although TP is clearly associated with angiogenesis, the exact link between TdR-related sugars and angiogenesis is still missing. Cells with a high TP can secrete angiogenic factors at high levels, including the vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), but the role of sugars in the activation of these factors remains unclear [5;13]. TdR-derived sugars accumulated in the cell fractions of the membrane and cytoskeleton, which may be related to glycation of proteins by the formation of AGE. This may lead to the hypothesis that protein glycation plays a role in the activation of the transcription of angiogenic factors, such as IL-8 and VEGF [13], which can subsequently be secreted. The secreted fraction consisted of non-proteins. This indicates that dR may be the main TdR-sugar-product that is secreted by TP-expressing cells. Possibly, protein-glycation of secreted proteins is not the main regulator of angiogenesis. Thus, glycation of proteins that remain intracellularly are possibly important for angiogenesis, by activating transcription of angiogenic enzymes. Since dR can be secreted by the cancer cells, it can be taken up by the endothelial cells, in which it can also undergo Schiff base reactions, forming AGE inside the endothelial cells, stimulating their angiogenic properties (Fig. 1).

A possible role of sugars in the angiogenic potential of TP was studied through its metabolite dR, which can stimulate endothelial cell migration and angiogenesis. dR is a strongly reducing sugar that can generate oxygen radical species during the early stages of protein glycation [13]. dR can bind to an amino group of proteins by a non-enzymatic reaction. This leads to the formation of a Schiff base, which can subsequently rearrange to an α-hydroxyketone. During this reaction, free oxygen radicals are produced. Thus, through the formation of dR, TP induces oxidative stress in TP-overexpressing tumor cells. This may result in the secretion of angiogenic factors, such as VEGF or IL-8 [13]. dR secreted by the cancer cells may in this way directly or indirectly (by its conversion to other sugars) stimulate angiogenesis. dR was secreted by the cancer cells in our study, indicating that dR can directly play a role in the angiogenic potential of TP.

In conclusion, dR-1-P and subsequently dR-5-P and dR are formed from TdR conversion by TP. Both metabolites may stimulate angiogenesis, e.g. by the stimulation of angiogenic factors, while secreted dR may attract endothelial cells to form new blood vessels in the tumor tissue. The sugars formed from TdR will accumulate in the membrane and cytoskeleton of the cells, indicating that the sugars are used for further cellular metabolism, possibly mediating angiogenesis in a more indirect manner.

Reference List

1. Bronckaers A, Gago F, Balzarini J, Liekens S. The dual role of thymidine phosphorylase in cancer development and chemotherapy. Med Res Rev 2009; 29(6):903-953.
2. Koukourakis MI, Giatromanolaki A, Kakolyris S, O'Byrne KJ, Apostolikas N, Skarlatos J et al. Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. Br J Cancer 1998; 77(10):1696-1703.
3. Liekens S, Bronckaers A, Perez-Perez MJ, Balzarini J. Targeting platelet-derived endothelial cell growth factor/thymidine phosphorylase for cancer therapy. Biochem Pharmacol 2007; 74(11):1555-1567.
4. de Bruin M, Temmink OH, Hoekman K, Pinedo HM, Peters GJ. Role of platelet derived endothelial cell growth factor/thymidine phosphorylase in health and disease. Cancer Therapy 2006;4:99-124.
5. Hotchkiss KA, Ashton AW, Schwartz EL. Thymidine phosphorylase and 2-deoxyribose stimulate human endothelial cell migration by specific activation of the integrins alpha 5 beta 1 and alpha V beta 3. J Biol Chem 2003; 278(21):19272-19279.
6. Hotchkiss KA, Ashton AW, Klein RS, Lenzi ML, Zhu GH, Schwartz EL. Mechanisms by which tumor cells and monocytes expressing the angiogenic factor thymidine phosphorylase mediate human endothelial cell migration. Cancer Res 2003; 63(2):527-533.
7. Ishikawa F, Miyazono K, Hellman U, Drexler H, Wernstedt C, Hagiwara K et al. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature 1989; 338(6216):557-562.
8. Seeliger H, Guba M, Koehl GE, Doenecke A, Steinbauer M, Bruns CJ et al. Blockage of 2-deoxy-D-ribose-induced angiogenesis with rapamycin counteracts a thymidine phosphorylase-based escape mechanism available for colon cancer under 5-fluorouracil therapy. Clin Cancer Res 2004; 10(5):1843-1852.
9. Moghaddam A, Zhang HT, Fan TP, Hu DE, Lees VC, Turley H et al. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc Natl Acad Sci U S A 1995; 92(4):998-1002.
10. Sengupta S, Sellers LA, Matheson HB, Fan TP. Thymidine phosphorylase induces angiogenesis in vivo and in vitro: an evaluation of possible mechanisms. Br J Pharmacol 2003; 139(2):219-231.
11. de BM, Smid K, Laan AC, Noordhuis P, Fukushima M, Hoekman K et al. Rapid disappearance of deoxyribose-1-phosphate in platelet derived endothelial cell growth factor/thymidine phosphorylase overexpressing cells. Biochem Biophys Res Commun 2003; 301(3):675-679.
12. Carta MC, Mattana A, Camici M, Allegrini S, Tozzi MG, Sgarrella F. Catabolism of exogenous deoxyinosine in cultured epithelial amniotic cells. Biochim Biophys Acta 2001; 1528(2-3):74-80.
13. Brown NS, Jones A, Fujiyama C, Harris AL, Bicknell R. Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors. Cancer Res 2000; 60(22):6298-6302.
14. Thornalley PJ, Wolff SP, Crabbe MJ, Stern A. The oxidation of oxyhaemoglobin by glyceraldehyde and other simple monosaccharides. Biochem J 1984; 217(3):615-622.
15. Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovasc Res 1998; 37(3):586-600.
16. Monnier VM. Nonenzymatic glycosylation, the Maillard reaction and the aging process. J Gerontol 1990; 45(4):B105-B111.
17. Wolff SP, Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem J 1987; 245(1):243-250.
18. Haraguchi M, Miyadera K, Uemura K, Sumizawa T, Furukawa T, Yamada K et al. Angiogenic

activity of enzymes. Nature 1994; 368(6468):198.
19. Stevenson DP, Milligan SR, Collins WP. Effects of platelet-derived endothelial cell growth factor/thymidine phosphorylase, substrate, and products in a three-dimensional model of angiogenesis. Am J Pathol 1998; 152(6):1641-1646.
20. de BM, van CT, Van der Born K, Kruyt FA, Fukushima M, Hoekman K et al. Role of platelet-derived endothelial cell growth factor/thymidine phosphorylase in fluoropyrimidine sensitivity. Br J Cancer 2003; 88(6):957-964.
21. Huck JH, Struys EA, Verhoeven NM, Jakobs C, van der Knaap MS. Profiling of pentose phosphate pathway intermediates in blood spots by tandem mass spectrometry: application to transaldolase deficiency. Clin Chem 2003; 49(8):1375-1380.
22. de BM, Peters GJ, Oerlemans R, Assaraf YG, Masterson AJ, Adema AD et al. Sulfasalazine down-regulates the expression of the angiogenic factors platelet-derived endothelial cell growth factor/thymidine phosphorylase and interleukin-8 in human monocytic-macrophage THP1 and U937 cells. Mol Pharmacol 2004; 66(4):1054-1060.
23. Laurensse EJ, Pinedo HM, Peters GJ. A sensitive non-radioactive assay for pyrimidine nucleoside phosphorylase using reversed-phase high performance liquid chromatography. Clin Chim Acta 1988; 178(1):71-78.
24. Wamelink MM, Struys EA, Huck JH, Roos B, van der Knaap MS, Jakobs C et al. Quantification of sugar phosphate intermediates of the pentose phosphate pathway by LC-MS/MS: application to two new inherited defects of metabolism. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 823(1):18-25.
25. Mathews ST, Plaisance EP, Kim T. Imaging systems for westerns: chemiluminescence vs. infrared detection. Methods Mol Biol 2009; 536:499-513.
26. Moriwaki Y, Yamamoto T, Yamakita J, Takahashi S, Higashino K. Comparative localization of aldehyde oxidase and xanthine oxidoreductase activity in rat tissues. Histochem J 1998; 30(2):69-74.
27. Sgarrella F, Del CA, Tozzi MG, Camici M. Deoxyribose 5-phosphate aldolase of Bacillus cereus: purification and properties. Biochim Biophys Acta 1992; 1118(2):130-133.
28. Cho SJ, Roman G, Yeboah F, Konishi Y. The road to advanced glycation end products: a mechanistic perspective. Curr Med Chem 2007; 14(15):1653-1671.
29. Brown NS, Streeter EH, Jones A, Harris AL, Bicknell R. Cooperative stimulation of vascular endothelial growth factor expression by hypoxia and reactive oxygen species: the effect of targeting vascular endothelial growth factor and oxidative stress in an orthotopic xenograft model of bladder carcinoma. Br J Cancer 2005; 92(9):1696-1701.
30. Tozzi MG, Camici M, Mascia L, Sgarrella F, Ipata PL. Pentose phosphates in nucleoside interconversion and catabolism. FEBS J 2006; 273(6):1089-1101.
31. Pieters R, Huismans DR, Loonen AH, Peters GJ, Hahlen K, van der Does-van den Berg et al. Adenosine deaminase and purine nucleoside phosphorylase in childhood lymphoblastic leukemia: relation with differentiation stage, in vitro drug resistance and clinical prognosis. Leukemia 1992; 6(5):375-380.
32. van Laarhoven JP, de Bruyn CH. Purine metabolism in relation to leukemia and lymphoid cell differentiation. Leuk Res 1983; 7(4):451-480.
33. Bardot V, Dutrillaux AM, Delattre JY, Vega F, Poisson M, Dutrillaux B et al. Purine and pyrimidine metabolism in human gliomas: relation to chromosomal aberrations. Br J Cancer 1994; 70(2):212-218.
34. Johnson MR, Diasio RB. Importance of dihydropyrimidine dehydrogenase (DPD) deficiency in patients exhibiting toxicity following treatment with 5-fluorouracil. Adv Enzyme Regul 2001; 41:151-157.
35. Thomas HR, Ezzeldin HH, Guarcello V, Mattison LK, Fridley BL, Diasio RB. Genetic regulation of dihydropyrimidinase and its possible implication in altered uracil catabolism. Pharmacogenet Genomics 2007; 17(11):973-987.

36. Westra JL, Hollema H, Schaapveld M, Platteel I, Oien KA, Keith WN et al. Predictive value of thymidylate synthase and dihydropyrimidine dehydrogenase protein expression on survival in adjuvantly treated stage III colon cancer patients. Ann Oncol 2005; 16(10):1646-1653.
37. Beck A, Etienne MC, Cheradame S, Fischel JL, Formento P, Renee N et al. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur J Cancer 1994; 30A(10):1517-1522.
38. Peters GJ, van Groeningen CJ, Pinedo HM. Dihydropyrimidine dehydrogenase in livers from mouse and rat, and in human liver, colon tumors, and mucosa in relation to anabolism of 5-fluorouracil. Adv Exp Med Biol 1998; 431:633-636.
39. Anan K, Mitsuyama S, Tamae K, Suehara N, Nishihara K, Ogawa Y et al. Increased dihydropyrimidine dehydrogenase activity in breast cancer. J Surg Oncol 2003; 82(3):174-179.
40. Shimoda M, Sawada T, Kubota K. Thymidylate synthase and dihydropyrimidine dehydrogenase are upregulated in pancreatic and biliary tract cancers. Pathobiology 2009; 76(4):193-198.
41. Bijnsdorp IV, Vrijland K, Vroling L, Fukushima M, Peters GJ. Increased migration by stimulation of thymidine phosphorylase in endothelial cells of different origin. Nucleosides Nucleotides Nucleic Acids 2010; 29(4-6):482-487.
42. Pula G, Mayr U, Evans C, Prokopi M, Vara DS, Yin X et al. Proteomics identifies thymidine phosphorylase as a key regulator of the angiogenic potential of colony-forming units and endothelial progenitor cell cultures. Circ Res 2009; 104(1):32-40.

Chapter 8

Summarizing discussion and future perspectives

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

Lung cancer is one of the deadliest diseases amongst the different types of cancer and the incidence is expected to rise globally. It is the second most common cancer in both men and women in the western world and is often detected at an advanced stage of disease. NSCLC represents around 80% of all lung cancers [1]. The current treatment of NSCLC patients has reached a plateau since efficacy of therapy is limited by resistance to conventional chemo- and radiotherapy. New therapies are therefore urgently needed to improve lung cancer survival. Genetic analyses of NSCLC has identified mutations in EGFR, p53, KRAS, BRAF, ERBB2, MET, STK11, PIK3CA and PARK2 genes. These mutations have led to new strategies, aiming at mutated protein targets, for example activating mutations of EGFR [1;2]. Targeting of the apoptotic machinery represents another novel approach and aims to selectively kill cancer cells by apoptosis activation while sparing normal cells. In this thesis the main focus was on exploring the use of the TRAIL receptor apoptotic pathway as a target for the treatment for NSCLC.

NON-CANONICAL SIGNALING BY TRAIL IN NSCLC CELLS

TRAIL is an interesting agent that induces apoptosis through the extrinsic pathway in tumor cells by binding to the death receptors TRAIL-R1 or TRAIL-R2. It has shown strong activity in vitro and in vivo either alone or as part of combination regimes [3]. Currently, different TRAIL receptor agonists have been developed such as recombinant (rh)TRAIL and receptor selective antibodies that are evaluated in phase II/III clinical trials in a wide variety of tumor types, including NSCLC [4]. The combined use of these agonists with other treatments has often been found to synergistically enhance anti-tumor activity or to overcome TRAIL resistance in preclinical studies, strategies that are currently explored in the clinic as well [5]. However, besides activating canonical caspase-dependent apoptosis via the TRAIL-specific death receptors, the same receptors can activate non-canonical cell survival or proliferation pathways in tumor cells, preventing effective therapy [6]. Recent studies even reported on metastases promoting activity of TRAIL as also outlined below. An overview of the currently known non-canonical TRAIL signals and their functional consequences in various tumor types is provided in Chapter 2. These include the activation of IκB/ NF-κB, MAPKs p38, JNK, ERK1/2, MAP3K, TAK1, PKC, PI3K/Akt and Src. The signals that stimulate invasive behavior of tumor cells, including those found in this thesis and as reported by others, such as Src, NF-κB and K-RAS are also discussed. In this thesis part of the work was dedicated to examine the possible counterproductive effects of TRAIL stimulation in NSCLC and to unravel underlying mechanisms of non-canonical kinases signaling.

In Chapter 3 we evaluated the kinetics of MAPK p38 and JNK upon TRAIL exposure in two different NSCLC cell lines and examined the effect on apoptosis activation. These kinases are known to be activated directly by TRAIL through the formation of a secondary complex consisting of the serine/threonine kinase RIP1, caspase-8 and FADD [7]. TRAIL treatment induced the phosphorylation of p38 and JNK1/2/3 only in sensitive H460 cells and not in the resistant A549 cell line. The use of chemical inhibitors and siRNAs indicated that JNK activation counteracted TRAIL-induced apoptosis, whereas activation of p38 stimulated apoptosis. RIP1 was cleaved following TRAIL treatment concomitantly with detectable JNK phosphorylation. Further examination of the role of RIP1 by short hairpin (sh)RNA-dependent knockdown showed that p38 can be phosphorylated in both RIP1-dependent and -independent manner, whereas JNK phosphorylation occurred independently of RIP1. The exact molecular mechanisms of p38 and JNK activation by TRAIL that occurs independently of RIP1 is yet unclear and remains to be unraveled. Immunoprecipitation experiments to determine the protein components that are responsible for the activation of these kinases will give us clues how to target these pathways more upstream. Inhibition of kinases that suppress apoptosis is expected to facilitate the elimination of cancer cells when combined with TRAIL receptor agonists. In addition, the activation of JNK by TRAIL occurred in a caspase-8-dependent manner, which was not the case for p38. Mcl-1 was shown to be a downstream target of both p38 and JNK and silencing of Mcl-1 with siRNA strongly augmented TRAIL-induced apoptosis in H460 cells. Taken together, our results suggest a model in which TRAIL-induced activation of p38 and JNK have counteracting effects on Mcl-1 expression leading to pro- or anti-apoptotic effects, respectively. The role of p38 and JNK in TRAIL signaling appears tumor-type dependent. In prostate cancer cells, for example, activation of both kinases prevented TRAIL-induced apoptosis [8]. JNK, on the other hand enhanced the apoptotic effect of TRAIL in human lymphoid cell lines [9]. This illustrates that caution is warranted when developing combination therapies with TRAIL receptor agonists and kinase inhibitors. Pre-selection of suitable patients groups and tumor type-dependent approaches will be required for optimal results in a clinical setting. Since Mcl-1 appears to be the main target of p38 and JNK, the combined use of TRAIL with a Mcl-1 inhibitor/antagonist, such as Gosypoll (AT-101) and GX15-070 could also provide an effective strategy. In colon, breast, and pancreatic cancer cells inhibition of Mcl-1 with these inhibitors increased TRAIL-induced apoptosis [10-12]. In NSCLC cells this remains to be formally demonstrated, although our data has shown that Mcl-1 knockdown sensitizes for TRAIL-dependent apoptosis (Chapter 3). Gosypoll and GX15-070 are both being evaluated in clinical trials (clinicaltrails.gov) and combined use with TRAIL might be interesting to explore in subsequent trials.

Recently, various different TRAIL receptor complexes have been identified that trigger alternative forms of cell death, including the necroptosome and ripoptosome [13]. Currently, the more precise mechanisms that trigger the various kinases pathways are elusive. Further research is required to determine the molecular mechanisms triggering and regulating non-canonical TRAIL signalling, which will also provide new clues for optimal use of TRAIL receptor agonists for the treatment of cancer.

An unexpected feature of TRAIL is the induction of migration and invasion in tumor cells, which has been shown previously in colon, pancreatic ductal adenocarcinoma, and cholangiocarcinoma cancer cells [14-16]. In Chapter 4 and as published [17], we describe TRAIL induced migration and invasion in NSCLC cells in a panel of NSCLC cell lines with different sensitivities for TRAIL. Migratory and invasive properties of TRAIL were observed in the resistant NSCLC cells, demonstrating possible pro-tumorigenic activity of TRAIL in this cancer type for the first time. Using a kinomic approach with PepChip peptide arrays we were able to identify kinases that were activated in TRAIL resistant A549 cells and not in sensitive H460 cells. Following validation of the hits, the Src-STAT3 pathway directly activated by TRAIL was found to be responsible for invasive behavior. Activation of Src could be linked with RIP1 since knockdown of RIP1 or chemical inhibition with necrostatin-1 prevented activation. In literature, several other mechanisms have been described underlying TRAIL-induced migration/invasion. In apoptosis resistant cholangiocarcinoma cancer cells migration was found to depend on NF-κB [15], and in colon cancer cells K-Ras, Raf-1 and the ROCK/LIM kinase/cofilin pathway were involved [14]. It would be interesting to study if the RIP1-Src-STAT3 pathway is also involved in TRAIL-induced migration in other tumor types as well. The migratory effect of TRAIL impedes its clinical value since unwanted pro-tumorigenic effects may occur when administered in patients. We postulate that better knowledge of these mechanisms may provide markers for selecting patients that will likely benefit from TRAIL-based therapy and will provide a rationalized basis for combination therapies with TRAIL agonists. Thus, kinases and their phosphorylated active forms may provide biomarkers to predict the sensitivity and/or consequences of TRAIL treatment. When required, TRAIL receptor agonists could be combined with small molecules that inhibit mediators of migration/invasion, such as Src, STAT3 or RIP1 inhibitors. In this context, markers for predicting TRAIL sensitivity in general would be of great therapeutic value. However, thus far only a few possible suitable biomarkers have been identified, which have not been tested in the clinic for upfront patient selection as yet. For example, TRAIL receptor O-glycosylation was reported to be a good predictor for TRAIL sensitivity [18]. O-linked glycosylation involves the binding of glycosyl groups to threonine and serine side chains leading to improved ligand binding and receptor protein activation. In cancer tissue specific O-glycosyltransferases were found to be overexpressed when compared to normal tissue and interestingly, were correlated with TRAIL sensitivity. In NSCLC, melanoma and pancreatic cancer cells, the expression of O-glycosylation initiating enzyme GALNT14 was

associated with TRAIL sensitivity [18]. Loss of cell surface expression of TRAIL-R1 and TRAIL-R2 by endocytosis was also found to be a biomarker for TRAIL resistance in breast cancer [19]. TRAIL sensitivity was recently reported to be predicted by a 71-gene signature as shown by a microarray study in a large number of different cell lines including NSCLC, leukemia, breast, renal, colon, melanoma, ovarian, prostate and CNS cancer [20]. Whether this gene signature will be of value for selecting patients for TRAIL therapy remains to be investigated. The next challenge will be to identify crucial targets and corresponding therapeutics from these biomarkers in order to determine the optimal TRAIL combination strategy. When possible samples obtained from both the primay tumor and metastatic lesions should be analyzed.

TRAIL COMBINATION APPROACHES FOR ENHANCING APOPTOSIS IN NSCLC

As mentioned earlier, combining TRAIL with other anti-cancer treatments can greatly increase the anti-tumor effect of TRAIL. This is illustrated by several studies in a wide range of different tumor types, whereby drugs like, proteasome inhibitors, HDAC and topoisomerase inhibitors increased TRAIL sensitivity or even sensitized resistant cells to TRAIL-induced apoptosis [21-25]. In Chapters 5 and 6 we describe the combination of TRAIL with two different anti-cancer agents; 17-AAG, a Hsp90 inhibitor, and trifluorothymidine (TFT), a thymidylate synthase inhibitor in NSCLC cells [26;27]. Both drugs showed synergy with TRAIL in a panel of NSCLC cells. Hsp90 is upregulated in many tumors and is thought to play an essential role in maintaining the malignant transformation of cancer cells, including by interaction and stabilizion of several key signalling proteins such as Akt, ErbB2, c-Met, and Raf-1. We found that 17-AAG increased TRAIL-induced apoptosis, mainly through the extrinsic pathway in A549 and H460 cells. Combined treatment resulted in cleavage of RIP1 and down-regulation of Akt and ERK. Inhibition of Akt activity by the chemical inhibitor LY294002 resulted in a significant increase in TRAIL-induced apoptosis. These results indicate that 17-AAG stimulates TRAIL-induced apoptosis in NSCLC via the extrinsic apoptotic pathway through down-regulation of Akt. As RIP1 is a client protein of Hsp90 and it was decreased in A549 cells, it is also likely to prevent RIP1-dependent non-canonical signaling and subsequent counterproductive effects. The inhibition of migration/invasion by 17-AAG has already been shown in glioma and breast cancer cells [28-30]. Even though the combination of 17-AAG and TRAIL proved to be quite effective in eradicating NSCLC cells, additional preclinical studies in mouse models will be required to further determine the value of the combination for possible translation to the clinic. Especially, because single agent studies with 17-AAG have shown limited activity in clinical trials [31], combination of this drug and TRAIL might be beneficial for cancer patients.

The other combination treatment being explored in this thesis was TRAIL together with TFT. TFT is part of the novel oral formulation TAS-102, which is currently being evaluated

in phase II clinical trials (clinicaltrials.gov). In this formulation, TFT is combined with a thymidine phosphorylase inhibitor (TPI) to increase the bioavailability of TFT. TFT interferes with thymidylate production and its triphosphate form is incorporated into the DNA, causing DNA damage. In Chapter 6, the combination TFT/TRAIL showed synergistic cytotoxicity in a panel of NSCLC cell lines, i.e. A549, H292, H322 and H460. The treatment affected cell cycle progression, with TRAIL inducing a G1 arrest and TFT a G2/M arrest. TFT activated Chk2 and reduced Cdc25c levels known to cause G2/M arrest. TRAIL-induced caspase-dependent apoptosis was enhanced by TFT, whereas TFT alone mainly induced caspase-independent cell death. Enhanced apoptosis correlated with the up-regulation of TRAIL-R2 surface expression, whereas TRAIL-R1 levels remained the same. TRAIL-R2 up-regulation was induced by p53, expression of which was also enhanced by TFT. Furthermore, the combination also caused a decrease in the expression of the anti-apoptotic proteins XIAP and $FLIP_L$. These proteins inhibit apoptosis and their overexpression has been associated with TRAIL resistance [32]. Thus, TFT can enhance TRAIL-induced apoptosis in NSCLC cells by sensitizing the extrinsic apoptotic pathway. Our findings suggest a possible therapeutic benefit of combined use of TFT and TRAIL in NSCLC. TFT is degraded effectively by thymidine phosphorylase (TP), making its half-life very short, only 12 minutes after injection [33]. TP is often overexpressed in cancer, including NSCLC, and potentially plays a role in the stimulation of angiogenesis [34;35]. The exact mechanism of angiogenesis induction is unclear, but is postulated to be related to thymidine-derived sugars. TP catalyzes the conversion of thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P), which can be converted to dR-5-P, glyceraldehyde-3-phosphate (G3P) or deoxyribose (dR). However, it was unclear which sugar accumulates in this reaction. In Chapter 7 [36], using liquid chromatography coupled to mass-spectometry (LC-MS-MS) we found that in TP-overexpressing cell lines, dR-1-P and dR-5-P accumulated intracellularly at high levels and dR was secreted extensively by the cells. A specific inhibitor of TP completely blocked TdR conversion, and indeed no sugars were formed. When further analyzing the subcellular localization of these sugars in (3)H-TdR cultured cells, TdR-derived sugars accumulated in the cytoskeleton and to some extent in the cell membrane, while incorporation into the DNA was responsible for trapping in the nucleus. Further studies should focus on which exact metabolic pathway is involved in the induction of angiogenesis, which likely involves more indirect mechanisms. It would also be interesting to study whether TP-overexpressing cells are sensitive for TFT after TP inhibition.

CONCLUSION

The TRAIL receptors have been convincingly demonstrated in preclinical models, including NSCLC, to be valid targets for the development of pro-apoptotic agonists. However, the differential sensitivity for apoptosis induction and even pro-tumorigenic effects in TRAIL resistant tumor cells requires a better understanding of the mechanisms that regulate TRAIL activity. Regardless of the more detailed molecular causes of the dichotomy in TRAIL signaling the combined use with standard anti-cancer drugs generally results in sensitization to TRAIL-induced apoptosis. Thus, possible unwanted effects of TRAIL treatment can be overcome by combination treatments, such as the ones we presented in this thesis. Elucidation of the molecular switches that determine the settings of the TRAIL pathway will help to develop targeted strategies to make the tumor cells apoptosis prone. Another challenge for the future is to identify biomarkers that allow the preselection of patients and determination of optimal TRAIL combination strategies in order to maximize therapeutic benefit.

Reference List

1. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359(13):1367-1380.
2. Gandara DR, Mack PC, Li T, Lara PN, Jr., Herbst RS. Evolving treatment algorithms for advanced non-small-cell lung cancer: 2009 looking toward 2012. Clin Lung Cancer 2009; 10(6):392-394.
3. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29(34):4752-4765.
4. Wu GS. TRAIL as a target in anti-cancer therapy. Cancer Lett 2009; 285(1):1-5.
5. Pore MM, Hiltermann TJ, Kruyt FA. Targeting apoptosis pathways in lung cancer. Cancer Lett 2010. http://dx.doi.org/10.1016/j.canlet.2010.09.012,
6. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39(7-8):1462-1475.
7. Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 2005; 280(49):40599-40608.
8. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional up-regulation of MCL-1. Cell Death Differ 2010; 17(8):1288-1301.
9. Herr I, Wilhelm D, Meyer E, Jeremias I, Angel P, Debatin KM. JNK/SAPK activity contributes to TRAIL-induced apoptosis. Cell Death Differ 1999; 6(2):130-135.
10. Huang S, Okumura K, Sinicrope FA. BH3 mimetic obatoclax enhances TRAIL-mediated apoptosis in human pancreatic cancer cells. Clin Cancer Res 2009; 15(1):150-159.
11. Kisim A, Atmaca H, Cakar B, Karabulut B, Sezgin C, Uzunoglu S et al. Pretreatment with AT-101 enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of breast cancer cells by inducing death receptors 4 and 5 protein levels. J Cancer Res Clin Oncol 2012.
12. Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB. Gossypol induces death receptor-5 through activation of the ROS-ERK-CHOP pathway and sensitizes colon cancer cells to TRAIL. J Biol Chem 2010; 285(46):35418-35427.
13. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M et al. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 2011; 43(3):449-463.
14. Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, Westendorp BF et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 2010; 138(7):2357-2367.
15. Ishimura N, Isomoto H, Bronk SF, Gores GJ. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 2006; 290(1):G129-G136.
16. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006; 25(56):7434-7439.
17. Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. J Cell Sci 2012.
18. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the pro-apoptotic ligand Apo2L/TRAIL. Nat Med 2007; 13(9):1070-1077.
19. Zhang Y, Zhang B. TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5. Mol Cancer Res 2008; 6(12):1861-1871.
20. Chen JJ, Knudsen S, Mazin W, Dahlgaard J, Zhang B. A 71-gene signature of TRAIL sensitivity in cancer cells. Mol Cancer Ther 2012; 11(1):34-44.
21. Dejosez M, Ramp U, Mahotka C, Krieg A, Walczak H, Gabbert HE et al. Sensitivity to

TRAIL/APO-2L-mediated apoptosis in human renal cell carcinomas and its enhancement by topotecan. Cell Death Differ 2000; 7(11):1127-1136.
22. Hougardy BM, Maduro JH, van der Zee AG, de Groot DJ, van den Heuvel FA, de Vries EG et al. Proteasome inhibitor MG132 sensitizes HPV-positive human cervical cancer cells to rhTRAIL-induced apoptosis. Int J Cancer 2006; 118(8):1892-1900.
23. Lacour S, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E et al. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. Oncogene 2003; 22(12):1807-1816.
24. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene 2004; 23(37):6261-6271.
25. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. Drug Resist Updat 2010; 13(1-2):2-15.
26. Altieri DC. Coupling apoptosis resistance to the cellular stress response: the IAP-Hsp90 connection in cancer. Cell Cycle 2004; 3(3):255-256.
27. Gallegos Ruiz MI, Floor K, Roepman P, Rodriguez JA, Meijer GA, Mooi WJ et al. Integration of gene dosage and gene expression in non-small cell lung cancer, identification of HSP90 as potential target. PLoS One 2008; 3(3):e0001722.
28. Kaur G, Belotti D, Burger AM, Fisher-Nielson K, Borsotti P, Riccardi E et al. Antiangiogenic properties of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin: an orally bioavailable heat shock protein 90 modulator. Clin Cancer Res 2004; 10(14):4813-4821.
29. Kim MS, Kwak HJ, Lee JW, Kim HJ, Park MJ, Park JB et al. 17-Allylamino-17-demethoxygeldanamycin down-regulates hyaluronic acid-induced glioma invasion by blocking matrix metalloproteinase-9 secretion. Mol Cancer Res 2008; 6(11):1657-1665.
30. Schulz R, Marchenko ND, Holembowski L, Fingerle-Rowson G, Pesic M, Zender L et al. Inhibiting the HSP90 chaperone destabilizes macrophage migration inhibitory factor and thereby inhibits breast tumor progression. J Exp Med 2012; 209(2):275-289.
31. Erlichman C. Tanespimycin: the opportunities and challenges of targeting heat shock protein 90. Expert Opin Investig Drugs 2009; 18(6):861-868.
32. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005; 12(3):228-237.
33. Ansfield FJ, Ramirez G. Phase I and II studies of 2'-deoxy-5-(trifluoromethyl)-uridine (NSC-75520). Cancer Chemother Rep 1971; 55(2):205-208.
34. Brockenbrough JS, Morihara JK, Hawes SE, Stern JE, Rasey JS, Wiens LW et al. Thymidine kinase 1 and thymidine phosphorylase expression in non-small-cell lung carcinoma in relation to angiogenesis and proliferation. J Histochem Cytochem 2009; 57(11):1087-1097.
35. Chujo M, Miura T, Kawano Y, Miyawaki M, Imakiire T, Hayashita Y et al. Thymidine phosphorylase levels and dihydropyrimidine dehydrogenase levels in non-small cell lung cancer tissues. Oncol Rep 2006; 16(4):777-780.
36. Bijnsdorp IV, Azijli K, Jansen EE, Wamelink MM, Jakobs C, Struys EA et al. Accumulation of thymidine-derived sugars in thymidine phosphorylase overexpressing cells. Biochem Pharmacol 2010; 80(6):786-792.

Chapter 9

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

INLEIDING

Longkanker is een van de meest voorkomende soorten kanker in de westerse wereld en is verantwoordelijk voor het grootste aantal kanker gerelateerde sterftegevallen. In de afgelopen decennia daalde de longkankersterfte bij mannen, terwijl het bij vrouwen juist toenam wat toegeschreven wordt aan een stijging van het aantal vrouwelijke rokers. Longkanker staat bekend om zijn sluipende karakter, omdat symptomen laat optreden en de ziekte in de meeste gevallen pas in een vergevorderd stadium wordt gevonden. Vanuit diagnostisch en behandelings perspectief wordt longkanker in twee groepen onderverdeeld, namelijk het kleincellige longcarcinoom, ongeveer 20% van alle longtumoren, en het niet-kleincellige longcarcinoom, dat in 80% van de gevallen voorkomt. Momenteel wordt het niet-kleincellig longcarcinoom via moleculaire diagnostiek in meerdere subtypes onderverdeeld, zoals mutaties in de Epidermal Growth Factor Receptor (EGFR), op grond waarvan besloten wordt om wel of niet een behandeling te geven met EGFR remmers. De beste behandeling voor niet-kleincellig longkanker in een niet-uitgezaaide vorm is een operatie, waarbij de gehele tumor wordt verwijderd. Wanneer de longtumor is uitgezaaid naar andere delen van het lichaam is het volledig operatief verwijderen niet meer mogelijk. In dit geval wordt de patiënt behandeld met chemotherapie en lokaal met radiotherapie bestraald om tumorcellen te doden, maar ook palliatief door hiermee bijvoorbeeld pijnklachten te bestrijden. Longkanker wordt gekenmerkt door grote moleculaire en cellulaire heterogeniteit en mede als gevolg hiervan zijn tumorcellen vaak resistent voor deze behandelingen of ontwikkelt resistentie zich tijdens de therapie hetgeen belangrijke oorzaken zijn voor de slechte prognose van longkanker. Bovendien zijn de behandelingen niet alleen cytotoxisch voor tumorcellen maar ook voor delende gezonde cellen. Hierdoor krijgen patiënten vaak last van vervelende bijwerkingen, zoals vermoeidheid, haaruitval en misselijkheid. Nieuwe behandelmethoden van longkanker zijn daarom noodzakelijk.

Het induceren van apoptose, geprogrammeerde celdood, selectief in tumorcellen en met behoud van normale cellen is een veelbelovende strategie in de behandeling van kanker. Dit kan gerealiseerd worden door het activeren van de zogenaamde Tumor Necrose Factor-gerelateerde apoptose-inducerende ligand (TRAIL) receptoren. In het laboratorium is aangetoond dat middelen die deze TRAIL receptoren activeren, zoals recombinant TRAIL en agonistische antilichamen, heel effectief tumorcellen doden, waaronder niet-kleincellige longtumorcellen. Echter een deel van de tumorcellen zijn TRAIL-resistent hetgeen voorkomen kan worden door TRAIL te combineren met chemo- en radio-therapeutica. Inmiddels zijn en worden er klinische studies uitgevoerd met TRAIL receptor agonisten tevens in combinatie met andere chemotherapieën in patiënten met verschillende tumor typen. Een eigenschap van TRAIL die tot dusver in een klein aantal

studies in het laboratorium gerapporteerd is, is dat het soms aanleiding kan geven tot groei van (tumor)cellen en het stimuleren van celmigratie en invasie. Dit ongewenste effect van TRAIL is nog niet goed bestudeerd en de moleculaire mechanismen hiervan zijn grotendeels onbekend.

In dit proefschrift hebben we de signalering van de TRAIL receptoren onderzocht in niet-kleincellig longkanker, waaronder de niet-apoptotische effecten zoals tumorcelmigratie en invasie in TRAIL resistente tumorcellen. Hierbij is vooral de rol van eiwit kinases bestudeerd, eiwitten die belangrijk zijn bij de transductie van signalen in de cel door het fosforyleren van substraten. Ook is de werking van een aantal nieuwe middelen onderzocht op het voorkomen van TRAIL resistentie en het celdodend effect van TRAIL te versterken.

EFFECTEN OP EIWIT KINASES

Een korte inleiding en een overzicht van de inhoud van dit proefschrift is te vinden in **hoofdstuk 1**. In **hoofdstuk 2** worden eiwit kinases die door TRAIL kunnen worden geactiveerd besproken. Dit zijn o.a. IκB, NF-κB, MAPKs, JNK, p38, ERK1/2, MAP3K, TAK1, PKC, PI3K/Akt en Src. De activatie van deze proteine kinases kunnen voor uiteenlopende effecten zorgen, zoals celoverleving, celdeling en uitzaaiing. De rol van Src hierbij is ontdekt in een studie beschreven in hoofdstuk 4 van dit proefschrift.

In **hoofdstuk 3** hebben we de kinase kinetiek en het effect op apoptose van TRAIL-geïnduceerde p38 en JNK activatie onderzocht in niet-kleincellige longkanker cellen met verschillende gevoeligheden voor het celdodend vermogen van TRAIL. Fosforylatie van deze twee eiwit kinases door TRAIL was waarneembaar in de apoptose gevoelige H460 cellijn en niet in de resistente A549 cellijn. De rol van p38 en JNK in TRAIL signalering werd bestudeerd d.m.v. selectieve eiwit kinase remmers en RNA interferentie (siRNA), waarmee de eiwitexpressie wordt verlaagd. Deze experimenten hebben aangetoond dat p38 nodig is voor TRAIL-geïnduceerde celdood in H460 cellen. Echter, activatie van JNK bleek TRAIL-geïnduceerde apoptose tegen te werken. Het onderliggende mechanisme van activatie en apoptose beïnvloeding is in meer detail onderzocht. Eerdere studies hadden aangetoond dat het eiwit kinase RIP1 betrokken is bij "de alternatieve TRAIL signalering", namelijk de activatie van eiwit kinases door dit ligand. Door gebruik te maken van short hairpin RNA tegen RIP1, hebben we laten zien dat p38 fosforylatie zowel RIP1 afhankelijk als onafhankelijk is; JNK fosforylatie daarentegen was RIP1 afhankelijk. Verder was caspase-8 noodzakelijk voor JNK activatie, maar speelde het geen rol in de fosforylering van p38. Het apoptose regulerend eiwit Mcl-1 kan apoptose remmen en bleek een downstream target te zijn van zowel p38 als JNK. De activatie van JNK door TRAIL bleek de expressie van Mcl-1 te verhogen, terwijl activatie van p38 door TRAIL juist de Mcl-1 expressie verlaagde, waaruit de tegenovergestelde effecten van p38 en JNK op apoptose te verklaren zijn. Activatie van JNK werd echter niet in alle onderzochte niet-kleincellige longkankercellen

waargenomen. Het gebruik van eiwit kinase remmers tegen JNK kan dus in sommige niet-kleincellige longkankercellen TRAIL apoptose versterken. Remmers van Mcl-1 in combinatie met TRAIL zouden dan een effectieve alternatieve strategie kunnen vormen voor het doden van niet-kleincellige longkankercellen.

Een onverwachte en weinig begrepen eigenschap van TRAIL is de inductie van migratie en invasie in tumorcellen. In **hoofdstuk 4** hebben we dit bestudeerd in niet-kleincellige longkanker cellijnen met verschillende gevoeligheden voor TRAIL. In TRAIL resistente niet-kleincellige longkanker cellen werd een migratie en invasie-stimulerend effect van TRAIL waargenomen. Met behulp van een 'proteine kinase profiling' techniek, die gebruik maakt van peptide microarrays zijn de eiwit kinases in kaart gebracht die door TRAIL geactiveerd worden in de resistente A549 cellijn, maar niet in apoptose gevoelige H460 cellen. Na het valideren van een aantal eiwit kinases hebben we ontdekt dat de Src-STAT3 pathway verantwoordelijk is voor TRAIL-geïnduceerde migratie en invasie. De activatie van deze route bleek RIP1 afhankelijk te zijn. Het verlagen van de expressie van dit eiwit met een short hairpin RNA en het remmen van RIP1 met necrostatin-1 zorgde er namelijk voor dat TRAIL niet meer in staat was Src en STAT3 te fosforyleren/ activeren. Op dit moment weten we niet of deze route ook in andere kankersoorten geactiveerd kan worden door TRAIL Echter, ongewenste effecten van TRAIL of TRAIL resistentie dienen in een therapeutische setting voorkomen te worden. Dit kan bereikt worden door, zoals eerder genoemd,TRAIL te combineren met andere geneesmiddelen, maar ook door toepassing van specifieke eiwit kinase remmers, zoals bijvoorbeeld Src en STAT3 remmers.

COMBINATIE STUDIES

In het tweede gedeelte van dit proefschrift is het mogelijke TRAIL apoptose versterkende effect van een aantal nieuwe antikankermiddelen onderzocht bij niet-kleincellige longtumorcellen. In **hoofdstuk 5** is het effect van een Hsp90 remmer, 17-AAG, op TRAIL apoptose onderzocht. Het Hsp90 eiwit speelt een rol bij de regulatie van verschillende eiwitten, waaronder de voor celdeling en celoverleving belangrijke eiwit kinases Akt, Erb2 en Raf-1. 17-AAG was in staat de TRAIL-geïnduceerde apoptose te versterken in de longkankercellen A549 en H460. Dit ging voornamelijk gepaard met een verhoogde activatie van de extrinsieke apoptose pathway. Co-incubatie met TRAIL en 17-AAG resulteerde in een toename van RIP1 afbraak en een verlaging van Akt en ERK expressies, eiwit kinases die celoverleving en proliferatie bevorderen. Inderdaad bleek het remmen van Akt met de chemische stof LY290042 de TRAIL-geïnduceerde apoptose te versterken. De Hsp90 remmer, 17-AAG blijkt dus een mogelijke kandidaat voor toepassingen in combinatie met TRAIL receptor agonisten voor een betere tumorceldoding.

Trifluorothymidine (TFT) is een nieuw chemotherapeuticum waarvan we het mogelijke TRAIL apoptose stimulerende effect onderzocht hebben in **hoofdstuk 6**. De chemische

structuur van TFT is gebaseerd op de bouwstenen van DNA met een toevoeging van extra fluorgroepen. TFT kan worden ingebouwd in het DNA en zorgen voor DNA schade met tumorceldood als gevolg. In een aantal niet-kleincellige longkankercellen was cytotoxische synergie te zien na TRAIL/TFT incubatie. De behandeling had een effect op de cel cyclus, waarbij TRAIL de cellen in de G1-fase remde en TFT in de G2/M fase. TFT activeerde het eiwit kinase Chk2 en verminderde de hoeveelheid Cdc25c, een eiwit dat cellen remt in de G2/M fase. TRAIL-geïnduceerde caspase afhankelijke apoptose werd versterkt door TFT terwijl TFT alleen ook voor caspase-onafhankelijke celdood zorgde. De versterkte inductie van apoptose kon gecorreleerd worden aan een toename van TRAIL receptor 2 (TRAIL-R2) expressie; TRAIL receptor 1 (TRAIL-R1) expressie daarentegen bleef onveranderd. Door de expressie van p53 te verlagen d.m.v. siRNA, hebben we kunnen aantonen dat de toename van TRAIL-R2 door TFT, p53 afhankelijk is. Verder zorgde de TFT/TRAIL combinatie ook voor een verlaging van de XIAP en $FLIP_L$ expressies. Deze eiwitten staan bekend om hun remmend effect op apoptose en TRAIL resistentie.

TFT wordt in hoge mate door het enzym thymidine phosphorylase (TP) afgebroken, waardoor de halfwaardetijd van TFT erg kort is, namelijk 12 minuten na intraveneuze toediening. TP komt in bepaalde tumoren sterk verhoogd tot expressie en wordt geacht een belangrijke rol te spelen in de stimulatie van angiogenese, de vorming van nieuwe bloedvaten uit bestaande bloedvaten. Het exacte mechanisme van angiogenese inductie door TP is echter niet helemaal duidelijk. Suikers die betrokken zijn bij de omzetting van thymidine naar thymine door TP spelen hoogstwaarschijnlijk een rol in dit proces. In **hoofdstuk 7** hebben we de specifieke suikers, dR-1-P en dR-5-P geïdentificeerd, die worden gevormd en uitgescheiden door cellen met hoge TP expressie. De suikers bleken voornamelijk aanwezig te zijn in het cytoskelet en in enige mate ook in het celmembraan. Dit geeft aan dat de suikers weer verder gemetaboliseerd kunnen worden in glycoproteinen (suiker-eiwitten), die belangrijk zijn voor celsignalering en celmotiliteit. Bovendien kunnen de suikers worden omgezet in zogenaamde advanced glycation endproducts (AGE), die een rol kunnen spelen in cel migratie. De suikers die door TP worden uitgescheiden zijn waarschijnlijk op een indirecte manier betrokken bij de inductie van angiogenese.

CONCLUSIE

Het activeren van de TRAIL receptoren, TRAIL-R1 en TRAIL-R2, met TRAIL receptor agonisten leidt tot apoptose, selectief in tumorcellen en lijkt een veelbelovende strategie voor de behandeling van kanker. Een onverwacht effect van TRAIL is het stimuleren van celgroei, celmigratie en celinvasief gedrag van tumorcellen, zoals hier nu ook aangetoond is in niet-kleincellige longkankercellen. Hierbij speelt activatie van bepaalde eiwit kinases waarschijnlijk een belangrijke rol, zoals Src en STAT3 welke invasief gedrag bleken te bevorderen. Remming van eiwit kinases is daarom een interessante benadering om het antitumor effect van TRAIL te versterken. Als alternatief kunnen andere chemotherapeutische middelen ingezet worden om TRAIL-geïnduceerde celdood te versterken en resistentie te niet te doen, zoals het hier gebruikte TFT of HSP90 remmers. Verder is de verwachting dat TRAIL therapie verder verbeterd kan worden door selectie van patiënten op basis van markers die de TRAIL gevoeligheid van tumorcellen kunnen voorspellen. De expressie en activiteit van specifieke eiwit kinases kunnen mogelijk als biomarker fungeren. Ten slotte, hebben we in dit proefschrift een aantal nieuwe pre-klinische inzichten verkregen die het gebruik van apoptose-inducerende medicijnen bij de behandeling van niet-kleincellig longkanker mogelijk kunnen verbeteren.

Dankwoord

Wat waren de afgelopen jaren geweldig, zeg! Mijn promotietraject was een mooie reis, waar ik ongelooflijk van heb genoten. Dit natuurlijk ook mede dankzij alle lieve mensen die hebben bijgedragen aan dit onderzoek, collega's, vrienden en familie. Vandaar dit hoofdstuk toegewijd aan jullie.

Allereerst wil ik God bedanken voor alle kracht en zegeningen. Ik dank Hem voor de persoon die ik ben en alles wat Hij op mijn pad heeft gestuurd. Ik houd van U.

Ik bedank mijn promotor, prof. dr. Frank A.E. Kruyt, degene die dit promotieonderzoek allemaal mogelijk heeft gemaakt. Lieve Frank, bedankt voor al je vertrouwen in mij, je hulp, inzet en de fijne begeleider die je bent. Eigenlijk vind ik het best jammer dat het nu echt afgelopen is, we vormen namelijk een Top Team!

Verder wil ook mijn andere promotor bedanken prof.dr. Godefridus. J. Peters. Beste Frits, hartelijk dank voor jouw flexibele instelling en dat ik altijd, maar dan ook echt altijd gewoon bij je binnen kon stormen. Ik vind het wel jammer dat ik niet in je nieuwe, dure auto mocht rijden. ☺

Tot slot mijn derde promotor, prof. dr. Steven de Jong. Beste Steven, hartelijk dank voor het kritische en snelle nakijken van al mijn stukken. En ook bedankt voor alle goede vragen die je tijdens de meetings stelde. Ik moest er in het begin wel aan wennen, maar ik snapte op een gegeven moment wel dat ik niet alle antwoorden gelijk paraat hoefde te hebben.

Ik bedank ook de beoordelingscommissie. Prof. dr. E.G.E de Vries, prof. dr. W. Quax en prof. dr. H. Groen voor het kritisch lezen van mijn proefschrift en uiteraard de goedkeuring!

Dit onderzoek was onderdeel van het Top Institute (TI) Pharma project: T3-112, "TNF ligands in cancer", wat de samenwerking tussen academici en de farmaceutische industrie bevordert. Alle leden binnen TI Pharma wil ik hartelijk danken, met name degenen die direct betrokken waren binnen dit project voor de interessante discussies en hun input tijdens de meetings.

Eén persoon wil ik wel in het bijzonder noemen, dr. Jos Joore. Lieve Jos, dank voor al je hulp met de PepChip experimenten. Ik heb zoveel bewondering voor de geweldige entrepreneur en de fijne persoon die je bent. Ondanks je drukke agenda, heb je toch tijd voor ons weten te maken om een hele dag ons het experiment te laten zien.

Met ons bedoel ik; ik en Henk Dekker. Lieve Henk, waar moet ik beginnen. Bedankt voor al je hulp, met in het begin de Pepchip experimenten. Daarna ook met alle andere dingen, de interessante discussies over kinases, layout van dit proefschrift, vragen over experimenten en

zelfs na werktijden (lees 23:45) kon ik je altijd bellen wanneer ik in een crisis zat! Ik heb zo met je gelachen en je bent echt van onschatbare waarde binnen de Medische Oncologie.

Twee collega's die met TRAIL werkten op het "TNF ligands in cancer" project, maar dan in Groningen wil ik ook bedanken. Dr. Annemieke Meijer, bedankt voor al je input tijdens de meetings en ondanks dat je zo druk was met het afronden van je promotieonderzoek destijds, was je ook aanwezig bij mijn werkbesprekingen, dat waardeer ik enorm. And of course dr. Saravanan Yuvaraj. Dear Saravanan, thanks for the amazing collaboration and all your contributions. We had soooo much fun, and I really loved my visits to Groningen!

Ook wil ik prof. dr Maikel Peppelenbosch bedanken voor zijn bijdrage aan Hoofdstuk 4. Beste Maikel, hartelijk dank voor je hulp en wat een eer dat we met jou als (o.a.) kinase expert konden samenwerken! Een andere geweldige onderzoeker met wie ik heb mogen samenwerken is dr. Tom Würdinger. Lieve Tom, dank voor je oprechte interesse, je briljante ideeën en alle positiviteit! Ik heb intens genoten van onze fijne samenwerking ☺.

I would also like to thank dr. Elisa Giovanetti. Dear Elisa, thank you so much for all your help, the great laughs, helpful discussions, and the mRNA experiments in Chapter 3 you did for me so fast. I really admire you how dedicated and the amazing researcher you are. Thanks for your amazing friendship!

I would also like to thank dr. Birgit Weyhenmeyer for her contribution to Chapter 2. Dear, Birgit, it's so funny …I haven't met you in real life yet, but I'm sure I would like you a lot! Good luck with the continuation of this project as a post-doc.

Dr. Irene Bijnsdorp wil ik ook uiteraard bedanken. Lieve Irene, heel veel dank voor je leuke samenwerking aan hoofdstukken 6 en 7. Ook dank voor het inwerken in het begin en al je hulp. Ik heb ook erg leuk samengewerkt met Ingrid van Roosmalen. Lieve Ingrid, je zit weliswaar in Groningen, maar we hebben het beste van weten te maken. Ik vond het zo gaaf! Dank voor al je receptor expressie experimenten, je weet hoeveel dat voor me betekend heeft. Heel veel succes met jouw promotieonderzoek.

Verder wil ik alle studenten bedanken voor hun buitengewone enthousiasme en inzet: Marzia, Nur, Jorn, Sjors, Koen, Meyram en Ellen.

Mijn Roomies van de gezelligste kamer (2.40) van het CCA! Henk, Maudy, Mariëtte, Kristy, Iris, Maarten, Maria en Jennifer. Wat hebben wij een leuke tijd gehad, zeg en wat ben ik dol op jullie! Ik hoop dat we nog lang contact kunnen houden. En zoals Kristy zei: "wij zijn toch je familie?!"….echt wel ☺

Alle collega's van de farmacologie, angiogenese, proteomics, dermatologie, radiotherapie, tumor profiling, longziekten, oncogenomics, medicinal chemistry, neuro-oncology, KNO,

pathologie, reumatologie, en immunotherapie wil ik bedanken voor hun gezelligheid en collegialiteit: Kees, Nienke, Elisa Paolicchi (lovely personality!) Clara, Auke, Elena (OMG, you are sooo cool!) Ietje, Richard Honeywell, Maha, Leticia, Amir, Mina, Bahar (jij bent een sterke meid, keep your head up), Laura (wat ben jij leuk!), Richard de Haas, Simone (een superwoman, zo behulpzaam), Saskia Broekman (love your style!), Kitty, Ed, Tse, Koen, Maaike, ChimpChimp, Yvette, Esther Kleibeuker (wij hebben gewoon een celebrity in onze groep ;-), Arjan, Roy, Rajshri, Patrycja (for all the hugs and kisses), Judy, Victor, en Stephanie (wat was jij een leuke studente, gezellig samen samba dansen in de celkweek!), Sander Piersma, Marc, Davide, Thang, Sander Spiekstra (bedankt voor je hulp met het FACS apparaat en dat ik in de kerstvakantie nog de laatste experimenten mocht doen), Ravi (het eiwitlab is nu zeker in goede handen), Peter, Francoise (altijd opgewekt!), Serge, Wouter (wat goed dat we nog wat van dat heerlijke ijs hebben kunnen scoren tijdens het longkanker congres), Ellen, Jantien, Ida, Saskia Nijmeijer (wauw, wat ben jij een energiebron, een chocolade lava met vanille ijs krijgt ons wel rustig), Oscar en Andrea (dank voor de gezellige FIGON dagen), Maikel, Herman, David, Mark (bedankt voor je fijne vriendschap en de geruststellingen dat het allemaal goed komt), Dennis, Esther, Lotte, Sjoerd, Michiel, Laurine, Jordi en…. Viola (you're one of a kind). Verder ook Maria Vosjan, Derrek en my favorite girls; Famke, Lotte, Renée en Sue Ellen, bedankt voor de gezellige "Fashion party" evening en wat zien jullie er altijd zo hip uit! Verder ook Gerrit, Joost, Jelle, Bas, Annelies, Saskia Santegoets, Dinja, Rieneke (bedankt voor je hulp met de AnnexinV/ apoptose assay), Sinéad en onze geweldige buurvrouw Tanja, ik kon altijd op je rekenen dat jij er nog was in de late avonduren! Ennee… sorry voor alle herrie (gegil, geschreeuw, gelach …etc) wat uit CCA2.40 kwam.

Ik wil ook graag het afdelingshoofd, prof. dr. Henk Verheul bedanken voor de stage die we in de kliniek mochten lopen. Zo heb ik kunnen zien waar we het allemaal voor doen. Het heeft me enorm geraakt en heel erg geïnspireerd, dank je wel, Henk. Ook wil ik prof. dr. Epie Boven bedanken, dat ik met haar poli mee mocht lopen. Ik heb hierdoor zoveel bewondering gekregen voor alle oncologen. Verder, wil ik (inmiddels medisch oncoloog) dr. Jens Voortman bedanken voor o.a. de gezelligheid tijdens het TAT congres. Wat een lieverd ben je toch ook, Jens. Ik snap helemaal waarom je zo ongelooflijk populair bent. Verder zonder twijfel mogelijk een van de tofste en leukste oncologen in het VUmc, dr. Hans van der Vliet. Beste Hans, jij bent het perfecte voorbeeld van iemand die een goede link vormt tussen de kliniek en het CCA.

Alle andere collega's uit de kliniek wil ik bedanken voor hun interesse, Maurice (ook een van mijn favoriete oncologen), Laura, Ariëtte, Marja, Vivianne, Marleen, Jamila, Chantal (jij bent absoluut zeker het zonnetje van de afdeling), Roel, Jantine, Margreet, Helen, Liesbeth, Stefan, Joyce, Marinel, Jacob en uiteraard Rita Ruijter. Lieve Rita, wat ben jij een schatje zeg, dank voor je oprechte hulp bij mijn toekomstplannen. Ik waardeer het enorm en ben je ontzettend dankbaar!

Verder wil ik ook dr. Eric Ronken bedanken. Lieve Eric, jij bent de doorslaggevende factor geweest dat ik gekozen heb met dit avontuur te beginnen. Ik ben en blijf je hier enorm dankbaar voor en erg leuk dat we nog contact hebben gehouden!

Mijn persoonlijke coach, Désirée Elderhorst wil ik ook hartelijk danken. Lieve Desirée, wij kenden elkaar al vanuit TI Pharma. Jij bent het perfecte voorbeeld van iemand die haar hart volgt. Ik heb ongelooflijk veel gehad aan jouw coaching en heb veel over mezelf geleerd. Heel veel succes met je praktijk!

Lieve tante Milly en mijn lieve 'geadopteerde zus' Sharda (ben dol op je aanstekelijke schaterlach ☺), jullie wil ik ook uit de bodem van hart bedanken. Superbedankt dat wij altijd welkom zijn en het is iedere keer weer feest met jullie!

Mijn lieve paranimfen, Mariëlle en Nasreen wat heb ik altijd een dolle pret met jullie. Lieve Mariëlle van CCA collega/kamergenootje naar beste vriendin. Echt zo leuk wat voor klik wij samen hebben en ik vind je echt een toppertje! Lieve Nasreen, mijn 'geadopteerde' peetmoeder, hoe lang kennen wij elkaar al…. ruim 10 jaar, hè! Ik ben zo blij dat ik altijd bij jou terecht kan en hoe bijzonder jij voor me bent.

Tot slot wil ik my Loved Ones bedanken; mijn lieve zus, Kaoutar, dank voor de inspiratiebron, die je bent geweest voor mij om met de studie Farmaceutische Wetenschappen te beginnen. Mijn lieve zusje Najoua, dank voor de gezellige ontbijtjes, waardoor ik mijn werkdagen met veel energie kon beginnen en het samen volgen van al onze favoriete series…. "You wanna be on top!". Mijn lieve broertje Iliass, je bent weliswaar de benjamin, maar wat ben jij belangrijk voor me, je wijsheid, je humor en alle zaken die je altijd weet te relativeren. Lieve pappie en mammie, dank voor alles wat jullie voor me hebben gedaan en alle vrijheden die jullie me hebben gegeven, waardoor ik de persoon ben geworden, die ik nu ben. My Loved Ones, Wat moet ik nou zonder jullie. Jullie zijn mijn steun en toeverlaat waar ik altijd op kan rekenen. Ik houd van jullie met heel mijn hart…

Love en Fierce,

Kaamar

CURRICULUM VITAE

Kaamar Azijli was born on 14 september 1984 in Amsterdam. In 2002 she obtained her VWO diploma at the Hervormd Lyceum West in Amsterdam. Subsequently, she started her study Pharmaceutical Sciences at the VU University in Amsterdam. During this study she completed several internships in the group of prof.dr. Rob Leurs (Medicinal Chemistry) about Histamine H_3 and H_4 receptors. She also did an internship in Solvay Pharmaceuticals B.V. (now Abbott Laboratories) in the group of dr. Eric Ronken. This internship was about the Dopamine D_2 receptor in the treatment of CNS diseases, such as parkinson and schizophrenia. Her literature study was written about gefitinib, the first EGFR inhibitor in the treatment of NSCLC under supervision of dr. Iwan de Esch (Medicinal Chemistry). She graduated on her Master Pharmaceutical Sciences in 2007. Next, in 2008 she started with her PhD project at the department of Medical Oncology at the VU University Medical Center in Amsterdam, entitled: "TRAIL-induced kinase activation and apoptosis: towards improved death receptor targeted therapy for lung cancer", which is described in this thesis.

———

(**In Dutch**)

Kaamar Azijli is geboren op 14 september 1984 te Amsterdam. In 2002 behaalde ze haar VWO diploma aan het Hervormd Lyceum West, te Amsterdam. Vervolgens begon ze haar studie Farmaceutische Wetenschappen aan de Vrije Universiteit in Amsterdam. Tijdens deze studie werden een aantal stages verricht in de groep van prof. dr. Rob Leurs (Medicinal Chemistry) over Histamine H_3 en H_4 receptoren. Ook heeft ze stage gelopen in het farmaceutisch bedrijf Solvay Pharmaceuticals B.V. (tegenwoordig Abbott Laboratories) in de groep van dr. Eric Ronken. Deze stage ging over de Dopamine D_2 receptor in de behandeling van ziektes in het centrale zenuwstelsel, zoals parkison en schizofrenie. Haar literatuurstudie schreef ze over gefitinib, de eerste EGFR remmer in de behandeling van niet-kleincellige longkanker onder begeleiding van dr. Iwan de Esch (Medicinal Chemistry). Haar Master Farmaceutische Wetenschappen rondde zij af in 2007. Het jaar daarop in 2008, begon ze aan haar promotie onderzoek aan het VU medisch centrum in Amsterdam getiteld: "TRAIL-induced kinase activation and apoptosis: towards improved death receptor targeted therapy for lung cancer", wat beschreven staat in dit proefschrift.